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NOGO RECEPTOR HOMOLOGS

FIELD OF THE INVENTION

5 The invention relates to neurology and molecular biology. More particularly, the invention relates to CNS neurons and axonal growth

BACKGROUND

10 Among the mechanisms through which the cells of an organism communicate with each other and obtain information and stimuli from their environment is through cell membrane receptor molecules expressed on the cell surface. Many such receptors have been identified, characterized, and sometimes classified into major receptor superfamilies based on structural motifs and signal transduction features. The receptors are a first essential link for translating an extracellular signal into a cellular
15 physiological response.

 Receptors on neurons are particularly important in the development of the nervous system during embryogenesis. The neurons form connections with target cells during development through axonal extension of the neurons toward the target cells in a receptor-mediated process. Axons and dendrites have a specialized region of their
20 distal tips known as the growth cone. Growth cones enable the neuron to sense the local environment through a receptor-mediated process and direct the movement of the axon or dendrite of the neuron toward the neuron's target cell. This process is known as elongation. Growth cones can be sensitive to several guidance cues, for example, surface adhesiveness, growth factors, neurotransmitters and electric fields. The
25 guidance of growth at the cone depends on various classes of adhesion molecules, intercellular signals, as well as factors that stimulate and inhibit growth cones.

 Interestingly, damaged neurons do not elongate in the central nervous system (CNS) following injury due to trauma or disease, whereas axons in the peripheral nervous system (PNS) regenerate readily. The fact that damaged CNS neurons fail to
30 elongate is not due to an intrinsic property of CNS axons, but rather due to the CNS environment that is not permissive for axonal elongation. Classical grafting experiments by Aguayo and colleagues (*e.g.*, Richardson *et al.*, (1980) *Nature* 284,

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264-265) demonstrated that CNS axons can in fact elongate over substantial distances within peripheral nerve grafts, and that CNS myelin inhibits CNS axon elongation. Therefore, given the appropriate environment, CNS axons can regenerate, implying that CNS axonal injury can potentially be addressed by appropriate manipulation of the
5 CNS environment.

The absence of axon regeneration following injury can be attributed to the presence of axon growth inhibitors. These inhibitors are predominantly associated with myelin and constitute an important barrier to regeneration. Axon growth inhibitors are present in CNS-derived myelin and the plasma membrane of
10 oligodendrocytes that synthesize myelin in the CNS (Schwab *et al.*, (1993) *Annu. Rev. Neurosci.* 16, 565-595). Myelin-associated inhibitors appear to be a primary contributor to the failure of CNS axon regeneration *in vivo* after an interruption of axonal continuity, whereas other non-myelin associated axon growth inhibitors in the CNS may play a lesser role. These inhibitors block axonal regeneration following
15 neuronal injury due to trauma, stroke or viral infection.

Numerous myelin-derived axon growth inhibitors have been characterized (see, for review, David *et al.*, (1999) WO995394547; Bandman *et al.*, (1999) U.S. Patent No. 5,858,708; Schwab, (1996) *Neurochem. Res.* 21, 755-761). Several components of CNS white matter, NI35, NI250 (Nogo) and Myelin-associated glycoprotein
20 (MAG), which have inhibitory activity for axonal extension, have been described as well (Schwab *et al.*, (1990) WO9005191; Schwab *et al.*, (1997) U.S. Patent No. 5,684,133). In particular, Nogo is a 250 kDa myelin-associated axon growth inhibitor that was originally characterized based on the effects of the purified protein *in vitro* and monoclonal antibodies that neutralize the protein's activity (Schwab (1990) *Exp.*
25 *Neurol.* 109, 2-5). The Nogo cDNA was first identified through random analysis of brain cDNA and had no suggested function (Nagase *et al.*, (1998) *DNA Res.* 5, 355-364). The identification of this Nogo cDNA as the cDNA encoding the 250 kDa myelin-associated axon growth inhibitor was discovered only recently (GrandPre *et al.*, (2000) *Nature* 403, 439-444; Chen *et al.*, (2000) *Nature* 403, 434-439; Prinjha *et al.*,
30 (2000) *Nature* 403, 383-384).

Importantly, Nogo has been shown to be the primary component of CNS myelin responsible for inhibiting axonal elongation and regeneration. Nogo's selective

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expression by oligodendrocytes and not by Schwann cells (the cells that myelinate P.S. axons) is consistent with the inhibitory effects of CNS myelin, in contrast to P.S. myelin (GrandPre *et al.*, (2000) *Nature* 403, 434-439). In culture, Nogo inhibits axonal elongation and causes growth cone collapse (Spillmann *et al.*, (1998) *J. Biol. Chem.* 272, 19283-19293). Antibodies (*e.g.*, IN-1) against Nogo have been shown to block most of the inhibitory action of CNS myelin on neurite growth *in vitro* (Spillmann *et al.*, (1998) *J. Biol. Chem.* 272:19283-19293). These experiments indicate that Nogo is the main component of CNS myelin responsible for inhibition of axonal elongation in culture. Furthermore, *in vivo*, the IN-1 antibody has been shown to enhance axonal regeneration after spinal cord injury, resulting in recovery of behaviors such as contact placing and stride length (Schnell and Schwab (1990) *Nature* 343, 269-272; Bregman *et al.*, (1995) *Nature* 378, 498-501). Thus, there is substantial evidence that Nogo is a disease-relevant molecular target. Agents that interfere with the binding of Nogo to its receptor would be expected to improve axonal regeneration in clinical states in which axons have been damaged, and improve patient outcome.

Modulation of Nogo has been described as a means for treatment of regeneration for neurons damaged by trauma, infarction and degenerative disorders of the CNS (Schwab *et al.*, (1994) WO9417831; Tatagiba *et al.*, (1997) *Neurosurgery* 40, 541-546) as well as malignant tumors in the CNS such as glioblastoma (Schwab *et al.*, (1993) U.S. Patent No. 5,250,414); Schwab *et al.*, (2000) U.S. Patent No. 6,025,333).

Antibodies which recognize Nogo have been suggested to be useful in the diagnosis and treatment of nerve damage resulting from trauma, infarction and degenerative disorders of the CNS (Schnell & Schwab, (1990) *Nature* 343, 269-272; Schwab *et al.*, (1997) U.S. Patent No. 5,684,133). For CNS axons, there is a correlation between the presence of myelin and the inhibition of axon regeneration over long distances (Savio and Schwab (1990) *Proc. Natl. Acad. Sci.* 87, 4130-4133; Keirstead *et al.*, (1992) *Proc. Natl. Acad. Sci.* 89, 11664-11668). After Nogo is blocked by antibodies, neurons can again extend across lesions caused by nerve damage (Schnell and Schwab (1990) *Nature* 343, 269-272).

SUMMARY OF THE INVENTION

Genes encoding homologs (NgR2 and NgR3) of a Nogo receptor (NgR1) in mice and humans have been discovered. Various domains in the polypeptides encoded by the NgR2 and NgR3 genes have been identified and compared to domains in mouse and human NgR1 polypeptides. This comparison has led to identification of a consensus sequence (NgR consensus sequence) that characterizes a family of proteins (NgR family). Based on these and other discoveries, the invention features molecules and methods for modulating axonal growth in CNS neurons.

The invention provides a polypeptide that contains a polypeptide containing a tryptophan rich LRRCT domain consisting of the amino acid sequence:

N X₁ W X₂ C X₃ C R A R X₄ L W X₅ W X₆ X₇ X₈ X₉ R X₁₀ S S S X₁₁ V

X₁₂ C X₁₃ X₁₄ P X₁₅ X₁₆ X₁₇ X₁₈ X₁₉ X₂₀ D L X₂₁ X₂₂ L X₂₃ X₂₄ X₂₅ D

X₂₆ X₂₇ X₂₈ C [SEQ ID NO. 19]

wherein X is any protein amino acid or a gap, and the polypeptide does not include amino acid sequence from residue 260 to 309 of SEQ ID NO: 5 (human NgR1) or SEQ ID NO: 17 (mouse NgR1).

Preferably, X17 and X23 are (independently) arginine or lysine. In some embodiments, the amino acid sequence of the LRRCT domain is residues 261-310 of SEQ ID NO:2, or residues 261-310 of SEQ ID NO: 2 with up to 10 conservative amino acid substitutions. In some embodiments, the polypeptide contains the following NTLRRCT amino acid sequence:

C P X₁ X₂ C X₃ C Y X₄ X₅ P X₆ X₇ T X₈ S C X₉ X₁₀ X₁₁ X₁₂ X₁₃ X₁₄ X₁₅ X₁₆ P
X₁₇ X₁₈ X₁₉ P X₂₀ X₂₁ X₂₂ X₂₃ R X₂₄ F L X₂₅ X₂₆ N X₂₇ I X₂₈ X₂₉ X₃₀ X₃₁ X₃₂ X₃₃
X₃₄ F X₃₅ X₃₆ X₃₇ X₃₈ X₃₉ X₄₀ X₄₁ X₄₂ L W X₄₃ X₄₄ S N X₄₅ X₄₆ X₄₇ X₄₈ I X₄₉
X₅₀ X₅₁ X₅₂ F X₅₃ X₅₄ X₅₅ X₅₆ X₅₇ L E X₅₈ L D L X₅₉ D N X₆₀ X₆₁ L X₆₂ X₆₃ X₆₄
X₆₅ P X₆₆ T F X₆₇ G L X₆₈ X₆₉ L X₇₀ X₇₁ L X₇₂ L X₇₃ X₇₄ C X₇₅ L X₇₆ X₇₇ L X₇₈
X₇₉ X₈₀ X₈₁ F X₈₂ G L X₈₃ X₈₄ L Q Y L Y L Q X₈₅ N X₈₆ X₈₇ X₈₈ X₈₉ L X₉₀ D

X₉₁ X₉₂ F X₉₃ D L X₉₄ N L X₉₅ H L F L H G N X₉₆ X₉₇ X₉₈ X₉₉ X₁₀₀ X₁₀₁ X₁₀₂
 X₁₀₃ X₁₀₄ F R G L X₁₀₅ X₁₀₆ L D R L L L H X₁₀₇ N X₁₀₈ X₁₀₉ X₁₁₀ X₁₁₁ V H X₁₁₂
 X₁₁₃ A F X₁₁₄ X₁₁₅ L X₁₁₆ R L X₁₁₇ X₁₁₈ L X₁₁₉ L F X₁₂₀ N X₁₂₁ L X₁₂₂ X₁₂₃ L
 X₁₂₄ X₁₂₅ X₁₂₆ X₁₂₇ L X₁₂₈ X₁₂₉ L X₁₃₀ X₁₃₁ L X₁₃₂ X₁₃₃ L R L N X₁₃₄ N X₁₃₅ W
 5 X₁₃₆ C X₁₃₇ C R X₁₃₈ R X₁₃₉ L W X₁₄₀ W X₁₄₁ X₁₄₂ X₁₄₃ X₁₄₄ R X₁₄₅ S S S X₁₄₆
 V X₁₄₇ C X₁₄₈ X₁₄₉ P X₁₅₀ X₁₅₁ X₁₅₂ X₁₅₃ X₁₅₄ X₁₅₅ D L X₁₅₆ X₁₅₇ L X₁₅₈ X₁₅₉ X₁₆₀
 D X₁₆₁ X₁₆₂ X₁₆₃ C [SEQ ID NO:18]

wherein X is any amino acid residue or a gap and wherein the polypeptide is not the
 10 polypeptide of SEQ ID NO: 5 (human NgR1) or SEQ ID NO: 17 (mouse NgR1). For
 example, X₆, X₃₇ and X₃₈ may represent a gap. Specific examples of polypeptides of
 the invention are SEQ ID NO: 2 (human NgR2), SEQ ID NO: 4 (mouse NgR3), and
 SEQ ID NO: 14 (human NgR3). In some embodiments, the polypeptide contains: (a)
 a NTLRRCT domain, and (b) less than a complete CTS domain, provided that a partial
 15 CTS domain, if present, consists of no more than the first 39 amino acids of the CTS
 domain. While the polypeptide may contain a functional GPI domain, a functional GPI
 domain may be absent, e.g., when a soluble polypeptide is desired. A polypeptide of
 the invention optionally includes an amino acid sequence of a heterologous
 polypeptide, e.g., an Fc portion of an antibody.

20 The invention also provides a nucleic acid encoding an above-described
 polypeptide; a vector containing the nucleic acid, which nucleic acid may be operably
 linked to an expression control sequence; and a transformed host cell containing the
 vector. A method of producing a polypeptide of the invention is also provided. The
 method includes introducing a nucleic acid encoding the above-described polypeptide
 25 into a host cell, culturing the cell under conditions suitable for expression of the
 polypeptide, and recovering the polypeptide.

The invention also provides an antisense molecule whose nucleotide sequence
 is complementary to a nucleotide sequence encoding a polypeptide selected from the
 group consisting of: a polypeptide consisting of residues 311-395 of SEQ ID NO: 2, a
 30 polypeptide consisting of residues 256-396 of SEQ ID NO:14 and a polypeptide
 consisting of residues 321-438 of SEQ ID NO: 4, wherein the nucleic acid is from 8 to

100 nucleotides in length, e.g., about 20, 30, 40, 50, 60, 70, 80 or 90 nucleotides. The invention also provides a nucleic acid encoding such an antisense molecule.

The invention also provides an antibody that binds to an above-described polypeptide. Polypeptides or antibodies of the invention can be formulated into
5 pharmaceutical compositions containing the polypeptide or antibody and a pharmaceutically acceptable carrier.

The invention also provides a method for decreasing inhibition of axonal growth of a CNS neuron. The method includes the step of contacting the neuron with an effective amount of a polypeptide or antibody of the invention.

10 The invention also provides a method for treating a central nervous system disease, disorder or injury. The method includes administering to a mammal, e.g., a human, an effective amount of a polypeptide or antibody of the invention. Exemplary diseases, disorders and injuries that may be treated using molecules and methods of the invention include, but are not limited to, cerebral injury, spinal cord injury, stroke,
15 demyelinating diseases, e.g., multiple sclerosis, monophasic demyelination, encephalomyelitis, multifocal leukoencephalopathy, panencephalitis, Marchiafava-Bignami disease, Spongy degeneration, Alexander's disease, Canavan's disease, metachromatic leukodystrophy and Krabbe's disease.

The invention also provides a method for identifying a molecule that binds a
20 polypeptide of the invention. The method includes the steps of: (a) providing a polypeptide of the invention; (b) contacting the polypeptide with the candidate molecule; and (c) detecting binding of the candidate molecule to the polypeptide.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the
25 invention belongs. In case of conflict, the present application, including definitions, will control. All publications, patent and other references mentioned herein are incorporated by reference.

The materials, methods and examples presented below are illustrative only, and not intended to be limiting. Other features and advantages of the invention will be
30 apparent from the detail description and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1A-1B shows an alignment of NgR2 (SEQ ID NO:2) and NgR3 (SEQ ID NO:4) with the known NgR, NgR1 (SEQ ID NO:5) and the Consensus Sequence (SEQ ID NO:6).

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Fig. 2. mNgR3 does not bind hNogoA(1055-1120). COS-7 cells were transfected with vectors encoding myc-NgR1 or myc-NgR3, fixed, and stained with anti-myc antibodies or AP-hNogoA(1055-1120).

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Fig.3. An alignment of the amino acid sequences of human NgR1, murine NgR1, murine NgR3, human NgR3 and human NgR2. Numbering begins with amino acid #1 of murine NgR3. The consensus sequence is listed below. The LRR NT domain is indicated by a shaded box; domains LLR 1, LLR 3, LLR 5, and LLR 7 are indicated by open boxes; LLR 2, LLR 4, LLR 6 and LLR 8 are indicated by shaded boxes; and the LLR CT domain is indicated by a shaded box. Amino acids in bold in LLR 8 indicate a conserved glycosylation sites. A dot indicates conserved cysteine residue in LRR4. Box at C terminus indicates putative GPI signals.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides purified and isolated polynucleotides (*e.g.*, DNA sequences and RNA transcripts, both sense and complementary antisense strands, both single- and double-stranded, including splice variants thereof) encoding NgR homologs, referred to herein as NgR. Unless indicated otherwise, as used herein, the abbreviation in lower case (NgR) refers to a gene, cDNA, RNA or nucleic acid sequence, whereas the upper case version (NgR) refers to a protein, polypeptide, peptide, oligopeptide, or amino acid sequence. Specific proteins are designated by number, *e.g.*, "NgR2" is a human NgR homolog, "NgR3" is a murine-derived NgR homolog, and "NgR1" is the known NgR identified by Dr. Stephen Strittmatter. Known NgRs are herein referred to as "NgRs." DNA polynucleotides of the invention include genomic DNA, cDNA and DNA that has been chemically synthesized in whole or in part.

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Standard reference works setting forth the general principles of recombinant DNA technology known to those of skill in the art include Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York (1998); Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, New York (1989); Kaufman *et al.*, Eds., HANDBOOK OF MOLECULAR AND CELLULAR METHODS IN BIOLOGY AND MEDICINE, CRC Press, Boca Raton (1995); McPherson, Ed., DIRECTED MUTAGENESIS: A PRACTICAL APPROACH, IRL Press, Oxford (1991).

As used herein, the term "axon" refers to a long cellular protrusion from a neuron, whereby action potentials are conducted, either to or from the cell body.

As used herein, the term "axonal growth" refers to an extension of the long process or axon, originating at the cell body and proceeded by the growth cone.

As used herein, the term "central nervous system disorder" refers to any pathological state associated with abnormal function of the central nervous system (CNS). The term includes, but is not limited to, altered CNS function resulting from physical trauma to cerebral tissue, viral infection, autoimmune mechanisms and genetic mutation.

As used herein, the term "demyelinating disease" refers to a pathological disorder characterized by the degradation of the myelin sheath of the oligodendrocyte cell membrane.

As used herein, the term "growth cone" refers to a specialized region at the tip of a growing neurite that is responsible for sensing the local environment and moving the axon toward its appropriate synaptic target cell.

As used herein, the term "growth cone movement" refers to the extension or collapse of the growth cone toward a neuron's target cell.

As used herein, the term "neurite" refers to a process growing out of a neuron. As it is sometimes difficult to distinguish a dendrite from an axon in culture, the term "neurite" is used for both.

As used herein, the term "oligodendrocyte" refers to a neuroglial cell of the CNS whose function is to myelinate CNS axons.

"Synthesized" as used herein and understood in the art, refers to polynucleotides produced by purely chemical, as opposed to enzymatic, methods.

"Wholly" synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means. By the term "region" is meant a physically contiguous portion of the primary structure of a biomolecule. In the case of proteins, a region is defined by a contiguous portion of the amino acid sequence of that protein. The term "domain" is herein defined as referring to a structural part of a biomolecule that contributes to a known or suspected function of the biomolecule. Domains may be co-extensive with regions or portions thereof; domains may also incorporate a portion of a biomolecule that is distinct from a particular region, in addition to all or part of that region. Examples of NgR protein domains include, but are not limited to, the signal peptide, extracellular (*i.e.*, N-terminal) domain, and leucine-rich repeat domains.

As used herein, the term "activity" refers to a variety of measurable indicia suggesting or revealing binding, either direct or indirect; affecting a response, *i.e.*, having a measurable affect in response to some exposure or stimulus, including, for example, the affinity of a compound for directly binding a polypeptide or polynucleotide of the invention, or, for example, measurement of amounts of upstream or downstream proteins or other similar functions after some stimulus or event. Such activities may be measured by assays such as competitive inhibition of NgR1 binding to Nogo assays wherein, for example, unlabeled, soluble NgR2 is added to an assay system in increasing concentrations to inhibit the binding of Nogo to NgR1 expressed on the surface of CHO cells. As another example, one may assess the ability of neurons to extend across lesions caused by nerve damage (as in Schnell and Schwab (1990) *Nature* 343, 269-272) following inhibition of Nogo by various forms of NgR2 and/or NgR3 as a biological indicator of NgR function.

As used herein, the term "antibody" is meant to refer to complete, intact antibodies, and Fab, Fab', F(ab)2, and other fragments thereof. Complete, intact antibodies include monoclonal antibodies such as murine monoclonal antibodies, chimeric antibodies, anti-idiotypic antibodies, anti-anti-idiotypic antibodies, and humanized antibodies.

As used herein, the term "binding" means the physical or chemical interaction between two proteins or compounds or associated proteins or compounds or

combinations thereof. Binding includes ionic, non-ionic, hydrogen bonds, Van der Waals, hydrophobic interactions, etc. The physical interaction, the binding, can be either direct or indirect, indirect being through or due to the effects of another protein or compound. Direct binding refers to interactions that do not take place through or
5 due to the effect of another protein or compound but instead are without other substantial chemical intermediates.

As used herein, the term "compound" means any identifiable chemical or molecule, including, but not limited to, small molecules, peptides, proteins, sugars, nucleotides or nucleic acids, and such compound can be natural or synthetic.

10 As used herein, the term "complementary" refers to Watson-Crick basepairing between nucleotide units of a nucleic acid molecule.

As used herein, the term "contacting" means bringing together, either directly or indirectly, a compound into physical proximity to a polypeptide or polynucleotide of the invention. The polypeptide or polynucleotide can be in any number of buffers,
15 salts, solutions etc. Contacting includes, for example, placing the compound into a beaker, microtiter plate, cell culture flask, or a microarray, such as a gene chip, or the like, which contains the nucleic acid molecule, or polypeptide encoding the NgR or fragment thereof.

As used herein, the phrase "homologous nucleotide sequence," or "homologous
20 amino acid sequence," or variations thereof, refers to sequences characterized by an identity at the nucleotide level, or a homology at the amino acid level, of at least the specified percentage. Homologous nucleotide sequences include those sequences coding for isoforms of proteins. Such isoforms can be expressed in different tissues of the same organism as a result of, for example, alternative splicing of RNA.

25 Alternatively, isoforms can be encoded by different genes. Homologous nucleotide sequences include nucleotide sequences encoding for a protein of a species other than humans, including, but not limited to, mammals. Homologous nucleotide sequences also include, but are not limited to, naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. A homologous nucleotide sequence does
30 not, however, include the nucleotide sequence encoding NgR1. Homologous amino acid sequences include those amino acid sequences which contain conservative amino acid substitutions and which polypeptides have the same binding and/or activity. A

homologous amino acid sequence does not, however, include the amino acid sequence encoding other known NgRs. Percent homology can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using the default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489, which is incorporated herein by reference in its entirety).

As used herein, the term "isolated" nucleic acid molecule refers to a nucleic acid molecule (DNA or RNA) that is substantially free of nucleic acids encoding other proteins with which it is associated in nature, i.e., a nucleic acid that has been removed from its native environment. Examples of isolated nucleic acid molecules include, but are not limited to, recombinant DNA molecules contained in a vector, recombinant DNA molecules maintained in a heterologous host cell, partially or substantially purified nucleic acid molecules, and synthetic DNA or RNA molecules. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated NgR nucleic acid molecule can contain less than about 50 kb, 25 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "heterologous" refers to a nucleotide or amino acid sequence that is a different, or non-corresponding sequence, or a sequence derived from a different species. For example, a mouse NgR nucleotide or amino acid sequence is heterologous to a human NgR nucleotide or amino acid sequence, and a human NgR nucleic or amino acid sequence is heterologous to a human immunoglobulin nucleotide or amino acid sequence.

As used herein, a "soluble NgR polypeptide" is a NgR polypeptide that does not anchor itself in a membrane. Such soluble polypeptides include, for example, NgR2 and NgR3 polypeptides that lack a sufficient portion of their GPI anchor signal

to anchor the polypeptide or are modified such that the GPI anchor signal is not adequate to result in replacement of the peptide with a GPI anchor. In preferred embodiments, up to 5, 10, 20 or 25 amino acids are removed from the C-terminus of NgR2 or NgR3 to make the respective proteins soluble. As used herein soluble NgR polypeptides include full-length or truncated (*e.g.*, with internal deletions) NgR.

Soluble NgR polypeptides may include the entire NgR protein up to the putative GPI signal sequence (*e.g.*, amino acid 1 to about amino acid 395 of NgR2, and from amino acid 1 to about amino acid 438 of NgR3). In other embodiments, the signal peptide of the proteins may be removed or truncated (*e.g.*, all or part of the signal sequence of NgR2, which spans amino acid 1 to about amino acid 30 of SEQ ID NO:2, may be removed; all or part of the signal sequence of NgR3, which spans amino acid 1 to about amino acid 40 of SEQ ID NO:4, may be removed). In some embodiments, the mature NgR2 (SEQ ID NO:8) and the mature NgR3 (SEQ ID NO:9) are used.

Soluble NgR polypeptides include at least one of the putative ligand-binding portions of NgR, including the first cysteine-rich region (SEQ ID NO:10, the leucine repeat region (SEQ ID NO:12) and the second cysteine-rich region (SEQ ID NO:11). In some embodiments, soluble NgR polypeptides consist of amino acid 1 through about amino acid 395 of SEQ ID NO:2, or amino acid 1 through about amino acid 438 of SEQ ID NO:4.

In other embodiments, the soluble NgR polypeptides are fusion proteins that contain amino acids 30 through about amino acid 395 of mature NgR2 or amino acid 40 through about amino acid 438 of NgR3, the C-terminal 10 amino acids of a human IgG 1 hinge region containing the two cysteine residues thought to participate in interchain disulfide bonding, and the CH2 and CH3 regions of a human IgG1 heavy chain constant domain. This type of recombinant protein is designed to modulate inhibition of axonal elongation through inhibition of the Nogo ligand binding to NgR1, or by inhibiting the ligand of the NgR from interacting with cell surface NgR. The NgR portion of the fusion binds to the Nogo ligand and the IgG1 portion binds to the FcγRI (macrophage) and FcγIII (NK cells and neutrophils) receptors.

The production of the soluble polypeptides useful in this invention may be achieved by a variety of methods known in the art. For example, the polypeptides may

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be derived from intact transmembrane NgR molecules by proteolysis using specific endopeptidases in combination with exopeptidases, Edman degradation, or both. The intact NgR molecule, in turn, may be purified from its natural source using conventional methods. Alternatively, the intact NgR may be produced by known recombinant DNA techniques using cDNAs, expression vectors and well-known techniques for recombinant gene expression.

Preferably, the soluble polypeptides useful in the present invention are produced directly, thus eliminating the need for an entire NgR as a starting material. This may be achieved by conventional chemical synthesis techniques or by well-known recombinant DNA techniques wherein only those DNA sequences which encode the desired peptides are expressed in transformed hosts. For example, a gene which encodes the desired soluble NgR polypeptide may be synthesized by chemical means using an oligonucleotide synthesizer. Such oligonucleotides are designed based on the amino acid sequence of the desired soluble NgR polypeptide. Specific DNA sequences coding for the desired peptide also can be derived from the full-length DNA sequence by isolation of specific restriction endonuclease fragments or by PCR synthesis of the specified region from cDNA.

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NOs:1, 3 or a complement of either of these nucleotide sequences, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or a portion of the nucleic acid sequences of SEQ ID NOs:1 or 3 as a hybridization probe, NgR nucleic acid sequences can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook *et al.*, eds., MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel, *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, NY, 1993).

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to NgR nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

As used herein, the terms "modulates" or "modifies" means an increase or decrease in the amount, quality, or effect of a particular activity or protein.

5 As used herein, the term "oligonucleotide" refers to a series of linked nucleotide residues which has a sufficient number of bases to be used in a polymerase chain reaction (PCR). This short sequence is based on (or designed from) a genomic or cDNA sequence and is used to amplify, confirm or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue.

10 Oligonucleotides comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 50 nucleotides, preferably about 15 to 30 nucleotides. They are chemically synthesized and may be used as probes.

 As used herein, the term "probe" refers to nucleic acid sequences of variable length, preferably between at least about 10 and as many as about 6,000 nucleotides, 15 depending on use. They are used in the detection of identical, similar or complementary nucleic acid sequences. Longer length probes are usually obtained from a natural or recombinant source, are highly specific and much slower to hybridize than oligomers. They may be single- or double-stranded and carefully designed to have specificity in PCR, hybridization membrane-based, or ELISA-like technologies.

20 The term "preventing" refers to decreasing the probability that an organism contracts or develops an abnormal condition.

 The term "treating" refers to having a therapeutic effect and at least partially alleviating or abrogating an abnormal condition in the organism.

 The term "therapeutic effect" refers to the inhibition or activation factors 25 causing or contributing to the abnormal condition. A therapeutic effect relieves to some extent one or more of the symptoms of the abnormal condition. In reference to the treatment of abnormal conditions, a therapeutic effect can refer to one or more of the following: (a) an increase in the proliferation, growth, and/or differentiation of cells; (b) inhibition (*i.e.*, slowing or stopping) of cell death; (c) inhibition of 30 degeneration; (d) relieving to some extent one or more of the symptoms associated with the abnormal condition; and (e) enhancing the function of the affected population

of cells. Compounds demonstrating efficacy against abnormal conditions can be identified as described herein.

5 The term "abnormal condition" refers to a function in the cells or tissues of an organism that deviates from their normal functions in that organism. An abnormal condition can relate to cell proliferation, cell differentiation, cell signaling, or cell survival. An abnormal condition may also include obesity, diabetic complications such as retinal degeneration, and irregularities in glucose uptake and metabolism, and fatty acid uptake and metabolism.

10 Abnormal cell proliferative conditions, for example, include cancers such as fibrotic and mesangial disorders, abnormal angiogenesis and vasculogenesis, wound healing, psoriasis, diabetes mellitus and inflammation.

 Abnormal differentiation conditions include, for example, neurodegenerative disorders, slow wound healing rates and slow tissue grafting healing rates.

15 Abnormal cell signaling conditions include, for example, psychiatric disorders involving excess neurotransmitter activity.

20 Abnormal cell survival conditions may also relate to conditions in which programmed cell death (apoptosis) pathways are activated or abrogated. A number of protein kinases are associated with the apoptosis pathways. Aberrations in the function of any one of the protein kinases could lead to cell immortality or premature cell death.

25 The term "administering" relates to a method of incorporating a compound into cells or tissues of an organism. The abnormal condition can be prevented or treated when the cells or tissues of the organism exist within the organism or outside of the organism. Cells existing outside the organism can be maintained or grown in cell culture dishes. For cells harbored within the organism, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral, dermal, injection, and aerosol applications. For cells outside of the organism, multiple techniques exist in the art to administer the compounds, including (but not limited to) cell microinjection techniques, transformation techniques and carrier techniques.

30 The abnormal condition can also be prevented or treated by administering a compound to a group of cells having an aberration in a signal transduction pathway to an organism. The effect of administering a compound on organism function can then

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be monitored. The organism is preferably a mouse, rat, rabbit, guinea pig or goat, more preferably a monkey or ape, and most preferably a human.

By "amplification" it is meant increased numbers of DNA or RNA in a cell compared with normal cells. "Amplification" as it refers to RNA can be the detectable
5 presence of RNA in cells, since in some normal cells there is no basal expression of RNA. In other normal cells, a basal level of expression exists, therefore in these cases amplification is the detection of at least 1–2-fold, and preferably more, compared to the basal level.

The amino acid sequences are presented in the amino to carboxy direction,
10 from left to right. The amino and carboxy groups are not presented in the sequence. The nucleotide sequences are presented by single strand only, in the 5' to 3' direction, from left to right. Nucleotides and amino acids are represented in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission or (for amino acids) by three letters code.

15

Nucleic Acids

Genomic DNA of the invention comprises the protein-coding region for a polypeptide of the invention and is also intended to include allelic variants thereof. It is widely understood that, for many genes, genomic DNA is transcribed into RNA
20 transcripts that undergo one or more splicing events wherein intron (*i.e.*, non-coding regions) of the transcripts are removed, or "spliced out." RNA transcripts that can be spliced by alternative mechanisms, and therefore be subject to removal of different RNA sequences but still encode a NgR polypeptide, are referred to in the art as splice variants which are embraced by the invention. Splice variants comprehended by the
25 invention therefore are encoded by the same original genomic DNA sequences but arise from distinct mRNA transcripts. Allelic variants are modified forms of a wild-type gene sequence, the modification resulting from recombination during chromosomal segregation or exposure to conditions which give rise to genetic mutation. Allelic variants, like wild-type genes, are naturally occurring sequences (as
30 opposed to non-naturally occurring variants arising from in vitro manipulation).

The invention also comprehends cDNA that is obtained through reverse transcription of an RNA polynucleotide encoding NgR (conventionally followed by

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second-strand synthesis of a complementary strand to provide a double-stranded DNA).

Preferred DNA sequences encoding a human NgR polypeptide is set out in SEQ ID NOs:1 and 13. A preferred DNA of the invention comprises a double
5 stranded molecule comprising the coding molecule (*i.e.*, the "coding strand") along with the complementary molecule (the "non-coding strand" or "complement") having a sequence unambiguously deducible from the coding strand according to Watson-Crick base-pairing rules for DNA. Also preferred are other polynucleotides encoding NgR polypeptides, as shown in SEQ ID NO:3, which comprises murine NgR homolog,
10 NgR3.

Also preferred are nucleotide sequences that encode at least a portion of a NgR polypeptide that has at least one biological function of a NgR. More preferred are nucleotide sequences that encode a portion of NgR that encodes at least the mature NgR without the hydrophobic C-terminal GPI signal. Also preferred are nucleotide
15 sequences that encode the portion of NgR that encodes at least the ligand-binding region of NgR.

The invention further embraces other species, preferably mammalian, homologs of the human NgR DNA. Species homologs, sometimes referred to as "orthologs," in general, share at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at
20 least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% homology with human DNA of the invention. Generally, percent sequence "homology" with respect to polynucleotides of the invention may be calculated as the percentage of nucleotide bases in the candidate sequence that are identical to nucleotides in the NgR sequences set forth in SEQ ID
25 NOs:1, 3 or 13, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

The polynucleotide sequence information provided by the invention makes possible large-scale expression of the encoded polypeptide by techniques well known and routinely practiced in the art. Polynucleotides of the invention also permit
30 identification and isolation of polynucleotides encoding related NgR polypeptides, such as human allelic variants and species homologs, by well-known techniques including Southern and/or Northern hybridization, and polymerase chain reaction (PCR).

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Examples of related polynucleotides include human and non-human genomic sequences, including allelic variants, as well as polynucleotides encoding polypeptides homologous to NgR and structurally related polypeptides sharing one or more biological, immunological, and/or physical properties of NgR. Non-human species genes encoding proteins homologous to NgR can also be identified by Southern and/or PCR analysis and are useful in animal models for NgR disorders. Knowledge of the sequence of a human NgR DNA also makes possible through use of Southern hybridization or polymerase chain reaction (PCR) the identification of genomic DNA sequences encoding NgR expression control regulatory sequences such as promoters, operators, enhancers, repressors, and the like. Polynucleotides of the invention are also useful in hybridization assays to detect the capacity of cells to express NgR. Polynucleotides of the invention may also provide a basis for diagnostic methods useful for identifying a genetic alteration(s) in a NgR locus that underlies a disease state or states, which information is useful both for diagnosis and for selection of therapeutic strategies.

The disclosure herein of a full-length polynucleotide encoding a NgR polypeptide makes readily available to the worker of ordinary skill in the art every possible fragment of the full-length polynucleotide. The invention, therefore, provides fragments of NgR-encoding polynucleotides comprising at least 6, and preferably at least 14, 16, 18, 20, 25, 50, or 75 consecutive nucleotides of a polynucleotide encoding NgR. Preferably, fragments of polynucleotides of the invention comprise sequences unique to the NgR-encoding polynucleotide sequence, and therefore hybridize under highly stringent or moderately stringent conditions only (*i.e.*, "specifically") to polynucleotides encoding NgR (or fragments thereof). Polynucleotide fragments of genomic sequences of the invention comprise not only sequences unique to the coding region, but also include fragments of the full-length sequence derived from introns, regulatory regions, and/or other non-translated sequences. Sequences unique to polynucleotides of the invention are recognizable through sequence comparison to other known polynucleotides, and can be identified through use of alignment programs routinely utilized in the art, *e.g.*, those made available in public sequence databases. Such sequences also are recognizable from Southern hybridization analyses to determine the number of fragments of genomic

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DNA to which a polynucleotide will hybridize. Polynucleotides of the invention can be labeled in a manner that permits their detection, including radioactive, fluorescent and enzymatic labeling.

5 Fragments of polynucleotides are particularly useful as probes for detection of full-length or fragment of NgR polynucleotides. One or more polynucleotides can be included in kits that are used to detect the presence of a polynucleotide encoding NgR, or used to detect variations in a polynucleotide sequence encoding NgR.

10 The invention also embraces DNAs encoding NgR polypeptides that hybridize under moderately stringent or high stringency conditions to the noncoding strand, or complement, of the polynucleotide in any of SEQ ID NOs:1 or 3.

Stringent conditions are known to those skilled in the art and can be found in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Preferably, the conditions are such that sequences at least about 65%, 70%, 75%, 85%, 90%, 95%, 98% or 99% homologous to each other typically remain
15 hybridized to each other. A non-limiting example of stringent hybridization conditions is hybridization in a high salt buffer comprising 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA and 500 mg/ml denatured salmon sperm DNA at 65°C. This hybridization is followed by one or more washes in 0.2X SSC, 0.01% BSA at 50°C. An isolated nucleic acid molecule of the invention that
20 hybridizes under stringent conditions to the sequence of SEQ ID NOs:1 or 3 corresponds to a naturally occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein). As used herein, "stringent hybridization conditions" means: 42°C in a hybridization
25 solution comprising 50% formamide, 1% SDS, 1 M NaCl, 10% (wt/vol) dextran sulfate, and washing twice for 30 minutes at 60°C in a wash solution comprising 0.1 X SSC and 1% SDS.

Vectors

30 Another aspect of the present invention is directed to vectors, or recombinant expression vectors, comprising any of the nucleic acid molecules described above. Vectors are used herein either to amplify DNA or RNA encoding NgR and/or to

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express DNA which encodes NgR. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of

5 vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction

10 into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used

15 interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), that serve equivalent functions.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with

20 vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: (1) to increase expression of recombinant protein; (2) to increase the solubility of the recombinant protein; and (3)

25 to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition

30 sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson (1988) *Gene* 67, 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia,

Piscataway, N.J.) that fuse glutathione-S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann *et al.*, (1988) *Gene* 69, 301-315) and pET 11d (Studier *et al.*, GENE
5 EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, CA. (1990) 60-89).

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in host bacteria with an impaired capacity to proteolytically cleave the recombinant protein. See, Gottesman, GENE EXPRESSION TECHNOLOGY:
10 METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, CA. (1990) 119-128. Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20, 2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by
15 standard DNA synthesis techniques.

In another embodiment, the NgR expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J.* 6, 229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30, 933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54, 113-123), pYES2
20 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

Alternatively, NgR can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, SF9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell. Biol.* 3,
25 2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170, 31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329, 840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6, 187-195). When used in mammalian cells, the
30 expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both

prokaryotic and eukaryotic cells. See, e.g., Chapters 16 and 17 of Sambrook *et al.*, (Eds.) MOLECULAR CLONING: A LABORATORY MANUAL. 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is
5 capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1, 268-277), lymphoid-specific promoters (Calame and Eaton
10 (1988) *Adv. Immunol.* 43, 235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8, 729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33, 729-740; Queen and Baltimore (1983) *Cell* 33, 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86, 5473-5477), pancreas-specific promoters (Edlund *et al.*
15 (1985) *Science* 230, 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss (1990) *Science* 249, 374-379) and the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3, 537-546).

20 The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner that allows for expression (by transcription of the DNA molecule) of an RNA molecule that is antisense NgR mRNA. Regulatory sequences operatively linked
25 to a nucleic acid cloned in the antisense orientation can be chosen that direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen that direct constitutive, tissue-specific or cell-type-specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid,
30 phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation

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of gene expression using antisense genes see Weintraub *et al.*, *Antisense RNA as a molecular tool for genetic analysis*, REVIEWS--TRENDS IN GENETICS, Vol. 1(1) 1986.

Preferred vectors include, but are not limited to, plasmids, phages, cosmids, episomes, viral particles or viruses and integratable DNA fragments (*i.e.*, fragments integratable into the host genome by homologous recombination). Preferred viral particles include, but are not limited to, adenoviruses, baculoviruses, parvoviruses, herpesviruses, poxviruses, adeno-associated viruses, Semliki Forest viruses, vaccinia viruses and retroviruses. Preferred expression vectors include, but are not limited to, pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). Other expression vectors include, but are not limited to, pSPORT™ vectors, pGEM™ vectors (Promega), pPROEXvectors™ (LTI, Bethesda, MD), Bluescript™ vectors (Stratagene), pQE™ vectors (Qiagen), pSE420™ (Invitrogen) and pYES2™ (Invitrogen).

Preferred expression vectors are replicable DNA constructs in which a DNA sequence encoding NgR is operably linked or connected to suitable control sequences capable of effecting the expression of the NgR in a suitable host. DNA regions are operably linked or connected when they are functionally related to each other. For example, a promoter is operably linked or connected to a coding sequence if it controls the transcription of the sequence. Amplification vectors do not require expression control domains, but rather need only the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants. The need for control sequences in the expression vector will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include, but are not limited to a transcriptional promoter, enhancers, an optional operator sequence to control transcription, polyadenylation signals, a sequence encoding suitable mRNA ribosomal binding and sequences which control the termination of transcription and translation. Such regulatory sequences are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as

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the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, NgR proteins, mutant forms of NgR, fusion proteins, etc.).

Preferred vectors preferably contain a promoter that is recognized by the host organism. The promoter sequences of the present invention may be prokaryotic, eukaryotic or viral. Examples of suitable prokaryotic sequences include the PR and PL promoters of bacteriophage lambda (THE BACTERIOPHAGE LAMBDA, Hershey, A.D. (Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1973), which is incorporated herein by reference in its entirety; LAMBDA II, Hendrix, R.W. (Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1980), which is incorporated herein by reference in its entirety); the *trp*, *recA*, heat shock, and *lacZ* promoters of *E. coli* and the SV40 early promoter (Benoist *et al.*, (1981) *Nature* 290, 304-310, which is incorporated herein by reference in its entirety). Additional promoters include, but are not limited to, mouse mammary tumor virus, long terminal repeat of human immunodeficiency virus, maloney virus, cytomegalovirus immediate early promoter, Epstein Barr virus, Rous sarcoma virus, human actin, human myosin, human hemoglobin, human muscle creatine and human metallothionein.

Additional regulatory sequences can also be included in preferred vectors. Preferred examples of suitable regulatory sequences are represented by the Shine-Dalgarno sequence of the replicase gene of the phage MS-2 and of the gene *cII* of bacteriophage lambda. The Shine-Dalgarno sequence may be directly followed by DNA encoding NgR and result in the expression of the mature NgR protein.

Moreover, suitable expression vectors can include an appropriate marker that allows the screening of the transformed host cells. The transformation of the selected host is carried out using any one of the various techniques well known to the expert in the art and described in Sambrook *et al.*, *supra*.

An origin of replication can also be provided either by construction of the vector to include an exogenous origin or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient. Alternatively, rather than using vectors

which contain viral origins of replication, one skilled in the art can transform mammalian cells by the method of co-transformation with a selectable marker and NgR DNA. An example of a suitable marker is dihydrofolate reductase (DHFR) or thymidine kinase (*see*, U.S. Patent No. 4,399,216).

5 Nucleotide sequences encoding NgR may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining and ligation with appropriate ligases. Techniques for such manipulation are disclosed by Sambrook *et al.*, *supra* and are well known in the art. Methods for construction of mammalian expression vectors are disclosed in, for example, Okayama *et al.*, (1983) *Mol. Cell. Biol.* 3:280, Cosman *et al.* (1986) *Mol. Immunol.* 23:935, Cosman *et al.*, (1984) *Nature* 312:768, EP-A-0367566, and WO 91/18982, each of which is incorporated herein by reference in its entirety.

15 **Host Cells and Transformed Host Cells**

According to another aspect of the invention, host cells are provided, including prokaryotic and eukaryotic cells, comprising a polynucleotide of the invention (or vector of the invention) in a manner that permits expression of the encoded NgR polypeptide. Preferably, the cell produces little or no endogenous NgR polypeptide. Polynucleotides of the invention may be introduced into the host cell as part of a circular plasmid, or as linear DNA comprising an isolated protein coding region or a viral vector. Methods for introducing DNA into the host cell that are well known and routinely practiced in the art include transformation, transfection, electroporation, nuclear injection, or fusion with carriers such as liposomes, micelles, ghost cells and protoplasts. Expression systems of the invention include bacterial, yeast, fungal, plant, insect, invertebrate, vertebrate and mammalian cells systems.

Host cells of the invention are a valuable source of immunogen for development of antibodies specifically immunoreactive with NgR. Host cells of the invention are also useful in methods for the large-scale production of NgR polypeptides wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells, or from the medium in which the cells

are grown, by purification methods known in the art, *e.g.*, conventional chromatographic methods including immunoaffinity chromatography, receptor affinity chromatography, hydrophobic interaction chromatography, lectin affinity chromatography, size exclusion filtration, cation or anion exchange chromatography, high pressure liquid chromatography (HPLC), reverse phase HPLC, and the like. Still other methods of purification include those methods wherein the desired protein is expressed and purified as a fusion protein having a specific tag, label or chelating moiety that is recognized by a specific binding partner or agent. The purified protein can be cleaved to yield the desired protein, or can be left as an intact fusion protein. Cleavage of the fusion component may produce a form of the desired protein having additional amino acid residues as a result of the cleavage process.

Knowledge of NgR DNA sequences allows for modification of cells to permit, or increase, expression of endogenous NgR. Cells can be modified (*e.g.*, by homologous recombination) to provide increased expression by replacing, in whole or in part, the naturally occurring NgR promoter with all or part of a heterologous promoter so that the cells express NgR at higher levels. The heterologous promoter is inserted in such a manner that it is operatively linked to endogenous NgR encoding sequences. (See, for example, PCT International Publication No. WO 94/12650, PCT International Publication No. WO 92/20808, and PCT International Publication No. WO 91/09955.) It is also contemplated that, in addition to heterologous promoter DNA, amplifiable marker DNA (*e.g.*, *ada*, *dhfr*, and the multifunctional CAD gene which encodes carbamoyl phosphate synthase, aspartate transcarbamylase, and dihydroorotase) and/or intron DNA may be inserted along with the heterologous promoter DNA. If linked to the NgR coding sequence, amplification of the marker DNA by standard selection methods results in co-amplification of the NgR coding sequences in the cells.

The DNA sequence information provided by the present invention also makes possible the development (*e.g.*, by homologous recombination or "knock-out" strategies; see Capecchi, *Science* 244:1288-1292 (1989)) of animals that fail to express functional NgR or that express a variant of NgR. Such animals (especially small laboratory animals such as rats, rabbits and mice) are useful as models for studying the *in vivo* activities of NgR and modulators of NgR.

Suitable host cells for expression of the polypeptides of the invention include, but are not limited to, prokaryotes, yeast, and eukaryotes. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequences. Suitable prokaryotic cells include, but are not limited to, bacteria of the genera *Escherichia*, *Bacillus*, *Salmonella*, *Pseudomonas*,
5 *Streptomyces* and *Staphylococcus*.

If a eukaryotic expression vector is employed, then the appropriate host cell would be any eukaryotic cell capable of expressing the cloned sequence. Preferably, eukaryotic cells are cells of higher eukaryotes. Suitable eukaryotic cells include, but are not limited to, non-human mammalian tissue culture cells and human tissue culture
10 cells. Preferred host cells include, but are not limited to, insect cells, HeLa cells, Chinese hamster ovary cells (CHO cells), African green monkey kidney cells (COS cells), human 293 cells, and murine 3T3 fibroblasts. Propagation of such cells in cell culture has become a routine procedure (*see*, Tissue Culture, Academic Press, Kruse
15 and Patterson, Eds. (1973), which is incorporated herein by reference in its entirety).

In addition, a yeast cell may be employed as a host cell. Preferred yeast cells include, but are not limited to, the genera *Saccharomyces*, *Pichia* and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Preferred yeast vectors can contain an origin of replication sequence from a 2T yeast plasmid, an autonomously
20 replication sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination and a selectable marker gene. Shuttle vectors for replication in both yeast and *E. coli* are also included herein.

Alternatively, insect cells may be used as host cells. In a preferred embodiment, the polypeptides of the invention are expressed using a baculovirus
25 expression system (*see*, Luckow *et al.*, *Bio/Technology*, 1988, 6, 47; BACULOVIRUS EXPRESSION VECTORS: A LABORATORY MANUAL, O'Rielly *et al.* (Eds.), W.H. Freeman and Company, New York, 1992; and U.S. Patent No. 4,879,236, each of which is incorporated herein by reference in its entirety). In addition, the MAXBAC™
30 complete baculovirus expression system (Invitrogen) can, for example, be used for production in insect cells.

Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, CA

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(1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin, dihydrofolate reductase (DHFR) and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding NgR or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

In a preferred embodiment, the polypeptides of the invention, including forms of NgR2 and NgR3, soluble forms of NgR, chimeric NgR polypeptides, NgR/Ig fusions and fragments and variations of each of the above are expressed in Chinese Hamster Ovary (CHO) cells.

In order to introduce the DNA fragment coding for the NgR protein or polypeptide into the CHO cell to express the recombinant NgR protein or polypeptide, it is necessary to construct the expression vector.

The vectors for CHO expression include, but are not limited to, pA1-11, pXT1, pRc/CMV, pRc/RSV and pcDNA1Neo. The promoter is not specifically limited

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provided it effectively promotes expression in CHO cells. Examples of suitable promoters are: SR α , SV40, LTR, CMV, and HSV-TK. Of these, CMV and Sr α promoters are preferred.

5 In addition to the above-mentioned promoters, the expression vectors may contain enhancers, splicing signals, polyadenylation signals, selectable markers and an SV40 replication origin. Suitable selectable markers include, but are not limited to the dihydrofolate reductase (DHFR) gene which provides resistance to methotrexate (MTX), the ampicillin resistance gene, and the neomycin resistance gene.

10 Examples of the expression vectors each containing the DNA coding for NgR, portions, fragments and soluble constructs thereof, include the vector (such as one described above), into which the promoter is operably linked (preferably upstream) to the nucleotide sequence encoding the desired NgR construct; a polyadenylation signal downstream from the nucleotide sequence encoding the NgR construct; and, preferably, the vector includes an operable DHFR gene. Preferably, the ampicillin
15 resistant gene is also operably contained in the vector.

CHO cell lacking the DHFR gene (Urlaub, G. *et al.*, (1980) *Proc. Natl. Acad. Sci. USA* 77, 4216-4220) and CHO-K1 (*Proc. Natl. Acad. Sci. USA* 60, 1275 (1968)) are suitable for use.

20 The NgR expression vectors prepared as above are introduced into CHO cells by any known method, including, but not limited to the calcium phosphate method (Graham and van der Eb (1973) *Virology* 52, 456-467) and electroporation (Nuemann *et al.*, (1982) *EMBO J.* 1, 841-845).

25 Transformants carrying the expression vectors are selected based on the above-mentioned selectable markers. Repeated clonal selection of the transformants using the selectable markers allows selection of stable cell lines having high expression of the NgR constructs. Increased MTX concentrations in the selection medium allows gene amplification and greater expression of the desired protein. The CHO cell containing the recombinant NgR can be produced by cultivating the CHO cells containing the NR expression vectors constitutively expressing the NgR constructs.

30 Media used in cultivating CHO cells includes DMEM medium supplemented with about 0.5 to 20% fetal calf serum, DMEM medium and RPMI1640 medium. The

pH of the medium is preferably about 6 to 8. Cultivation is preferably at about 30 to 40°C for about 15 to 72 hours with aeration.

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) NgR protein. Accordingly, the invention further provides methods for producing NgR protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding NgR has been introduced) in a suitable medium such that NgR protein is produced. In another embodiment, the method further comprises isolating NgR from the medium or the host cell.

In situations where the NgR polypeptide will be found primarily intracellularly, intracellular material (including inclusion bodies for Gram-negative bacteria) can be extracted from the host cell using any standard technique known to one of ordinary skill in the art. Such methods would encompass, by way of example and not by way of limitation, lysing the host cells to release the contents of the periplasm/cytoplasm by French press, homogenization and/or sonication followed by centrifugation.

If the NgR polypeptide has formed inclusion bodies in the cytosol, such inclusion bodies may frequently bind to the inner and/or outer cellular membranes. Upon centrifugation, the inclusion bodies will be found primarily in the pellet material. The pellet material can then be treated at pH extremes or with one or more chaotropic agents such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris-carboxyethyl phosphine at acid pH to release, break apart and solubilize the inclusion bodies. Once solubilized, NgR polypeptide can be analyzed using gel electrophoresis, immunoprecipitation or the like. Various methods of isolating the NgR polypeptide would be apparent to one of ordinary skill in the art, for example, isolation may be accomplished using standard methods such as those set forth below and in Marston *et al* (1990) *Meth. Enzymol.* 182, 264-275 (incorporated by reference herein in its entirety).

If isolated NgR polypeptide is not biologically active following the isolation procedure employed, various methods for "refolding" or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological

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activity. Methods known to one of ordinary skill in the art include adjusting the pH of the solubilized polypeptide to a pH usually above 7 and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for inclusion body solubilization but usually at a lower concentration and is not necessarily the same chaotrope as used for the solubilization. It may be required to employ a reducing agent or the reducing agent plus its oxidized form in a specific ratio, to generate a particular redox potential allowing for disulfide shuffling to occur in the formation of the protein's cysteine bridge(s). Some of the commonly used redox couples include cysteine/cystamine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol (DTT)/dithiane DTT, 2-mercaptoethanol (bME)/dithio-b(ME). To increase the efficiency of the refolding, it may be necessary to employ a cosolvent, such as glycerol, polyethylene glycol of various molecular weights and arginine.

Transgenic Animals

The host cells of the invention can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which NgR-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous NgR sequences have been introduced into their genome or homologous recombinant animals in which endogenous NgR sequences have been altered. Such animals are useful for studying the function and/or activity of NgR and for identifying and/or evaluating modulators of NgR activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA that is integrated into the genome of a cell from which a transgenic animal develops and that remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous NgR gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA

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molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

5 A transgenic animal of the invention can be created by introducing NgR-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The human NgR DNA sequence of SEQ ID NOs:1 or 3 can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homolog of the human NgR gene, such as a mouse NgR gene, can be isolated based on hybridization to the human NgR cDNA (described
10 further above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the NgR transgene to direct expression of NgR protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly
15 animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866; 4,870,009; and 4,873,191; and Hogan 1986, in MANIPULATING THE MOUSE EMBRYO, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the
20 NgR transgene in its genome and/or expression of NgR mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding NgR can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which
25 contains at least a portion of a NgR gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the NgR gene. The NgR gene can be a human gene (*e.g.*, SEQ ID NOs:1 or 13), but more preferably, is a non-human homolog of a human NgR gene. For example, a mouse homolog of human NgR gene of SEQ ID NOs:1 or 13 can be used to construct a homologous
30 recombination vector suitable for altering an endogenous NgR gene in the mouse genome. In one embodiment, the vector is designed such that, upon homologous

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recombination, the endogenous NgR gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector).

Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous NgR gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous NgR protein). In the homologous recombination vector, the altered portion of the NgR gene is flanked at its 5' and 3' ends by additional nucleic acid of the NgR gene to allow for homologous recombination to occur between the exogenous NgR gene carried by the vector and an endogenous NgR gene in an embryonic stem cell. The additional flanking NgR nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector. See *e.g.*, Thomas *et al.* (1987) *Cell* 51:503 for a description of homologous recombination vectors. The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced NgR gene has homologously recombined with the endogenous NgR gene are selected (see *e.g.*, Li *et al.* (1992) *Cell* 69:915).

The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras. See *e.g.*, Bradley 1987, In: TERATOCARCINOMAS AND EMBRYONIC STEM CELLS: A Practical Approach, Robertson, ed. IRL, Oxford, pp. 113-152. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley (1991) *Curr. Opin. Biotechnol.* 2:823-829; PCT International Publication Nos.: WO 90/11354; WO 91/01140; WO 92/0968; and WO 93/04169.

In another embodiment, transgenic non-humans animals can be produced that contain selected systems that allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For

a description of the cre/loxP recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a cre/loxP recombinase system is used to regulate

5 expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

10 Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut *et al.* (1997) *Nature* 385:810-813. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G₀ phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal

15 of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

Antisense

Also provided by the invention are antisense polynucleotides that recognize and hybridize to NgR polynucleotides. Full-length and fragment antisense polynucleotides are provided. Fragment antisense molecules of the invention include (i) those that

25 specifically recognize and hybridize to NgR RNA (as determined by sequence comparison of DNA encoding NgR to DNA encoding other known molecules). Identification of sequences unique to NgR encoding polynucleotides can be deduced through use of any publicly available sequence database, and/or through use of commercially available sequence comparison programs. After identification of the

30 desired sequences, isolation through restriction digestion or amplification using any of the various polymerase chain reaction techniques well known in the art can be

performed. Antisense polynucleotides are particularly relevant to regulating expression of NgR by those cells expressing NgR mRNA.

Antisense oligonucleotides, or fragments of a nucleotide sequence set forth in SEQ ID NO:1, 3, 13 or sequences complementary or homologous thereto, derived from the nucleotide sequences of the present invention encoding NgR are useful as diagnostic tools for probing gene expression in various tissues. For example, tissue can be probed *in situ* with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques to investigate native expression of this enzyme or pathological conditions relating thereto. In specific aspects, antisense nucleic acid molecules are provided that comprise a sequence complementary to at least about 10, 25, 50, 100, 250 or 500 nucleotides or an entire NgR coding strand, or to only a portion thereof. Nucleic acid molecules encoding fragments, homologs, derivatives and analogs of a NgR protein of SEQ ID NO:2, 4 or 14 or antisense nucleic acids complementary to a NgR nucleic acid sequence of SEQ ID NOs:1, 3 or 13 are additionally provided.

In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding NgR. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues (*e.g.*, the protein coding region of human NgR corresponds to the coding region SEQ ID NO:1, 3 or 13). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding NgR. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

Antisense oligonucleotides are preferably directed to regulatory regions of a nucleotide sequence of SEQ ID NO:1, 3, 13 or mRNA corresponding thereto, including, but not limited to, the initiation codon, TATA box, enhancer sequences, and the like. Given the coding strand sequences encoding NgR disclosed herein (*e.g.*, SEQ ID NO:1, 3 or 13), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick or Hoogsteen base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of NgR mRNA, but more preferably is an oligonucleotide that is antisense to only a portion of the coding

or noncoding region of NgR mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of NgR mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis or enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used.

Examples of modified nucleotides that can be used to generate the antisense nucleic acid include: 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention (preferably oligonucleotides of 10 to 20 nucleotides in length) are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA

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and/or genomic DNA encoding a NgR protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. Suppression of NgR expression at either the transcriptional or translational level is useful to generate cellular or animal models for diseases/conditions characterized by aberrant NgR expression. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule that binds to DNA duplexes, through specific interactions in the major groove of the double helix.

Phosphorothioate and methylphosphonate antisense oligonucleotides are specifically contemplated for therapeutic use by the invention. The antisense oligonucleotides may be further modified by adding poly-L-lysine, transferrin polylysine or cholesterol moieties at their 5' end.

An example of a route of administration of antisense nucleic acid molecules of the invention includes direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies that bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier *et al.*, (1987) *Nucleic Acids Res.* 15, 6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.*, (1987) *Nucleic Acids Res.* 15, 6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.*, (1987) *FEBS Lett.* 215, 327-330).

The NgR sequences taught in the present invention facilitate the design of novel transcription factors for modulating NgR expression in native cells and animals,

and cells transformed or transfected with NgR polynucleotides. For example, the Cys₂-His₂ zinc finger proteins, which bind DNA via their zinc finger domains, have been shown to be amenable to structural changes that lead to the recognition of different target sequences. These artificial zinc finger proteins recognize specific target sites with high affinity and low dissociation constants, and are able to act as gene switches to modulate gene expression. Knowledge of the particular NgR target sequence of the present invention facilitates the engineering of zinc finger proteins specific for the target sequence using known methods such as a combination of structure-based modeling and screening of phage display libraries (Segal *et al.*, (1999) *Proc. Natl. Acad. Sci. USA* 96, 2758-2763; Liu *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 94, 5525-5530; Greisman *et al.* (1997) *Science* 275, 657-661; Choo *et al.*, (1997) *J. Mol. Biol.* 273, 525-532). Each zinc finger domain usually recognizes three or more base pairs. Since a recognition sequence of 18 base pairs is generally sufficient in length to render it unique in any known genome, a zinc finger protein consisting of 6 tandem repeats of zinc fingers would be expected to ensure specificity for a particular sequence (Segal *et al.*, (1999), above). The artificial zinc finger repeats, designed based on the promoter of NgR sequences, are fused to activation or repression domains to promote or suppress NgR expression (Liu *et al.*, (1997), above). The promoter of NgR may be obtained by standard methods known to one of ordinary skill in the art with the disclosure contained herein and knowledge of the NgR sequence. Alternatively, the zinc finger domains can be fused to the TATA box-binding factor (TBP) with varying lengths of linker region between the zinc finger peptide and the TBP to create either transcriptional activators or repressors (Kim *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 94, 3616-3620. Such proteins and polynucleotides that encode them, have utility for modulating NgR expression *in vivo* in both native cells, animals and humans; and/or cells transfected with NgR-encoding sequences. The novel transcription factor can be delivered to the target cells by transfecting constructs that express the transcription factor (gene therapy), or by introducing the protein. Engineered zinc finger proteins can also be designed to bind RNA sequences for use in therapeutics as alternatives to antisense or catalytic RNA methods (McColl *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 96, 9521-9526; Wu *et al.*, (1995) *Proc. Natl. Acad. Sci. USA* 92, 344-348). The present invention contemplates methods of

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designing such transcription factors based on the gene sequence of the invention, as well as customized zinc finger proteins, that are useful to modulate NgR expression in cells (native or transformed) whose genetic complement includes these sequences.

5 **Ribozymes and PNA moieties**

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity that are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes,
10 described in Haselhoff and Gerlach (1988) *Nature* 334, 585-591) can be used to catalytically cleave NgR mRNA transcripts to thereby inhibit translation of NgR mRNA. A ribozyme having specificity for a NgR-encoding nucleic acid can be designed based upon the nucleotide sequence of a NgR DNA disclosed herein (*i.e.*, SEQ ID NOs:1, 3 or 13). For example, a derivative of a Tetrahymena L-19 IVS RNA
15 can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a NgR-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, NgR mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel *et al.*,
20 (1993) *Science* 261, 1411-1418.

Alternatively, NgR gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the NgR (*e.g.*, the NgR promoter and/or enhancers) to form triple helical structures that prevent transcription of the NgR gene in target cells. See generally, Helene (1991) *Anticancer Drug Des.* 6:
25 569-584; Helene. *et al.*, (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *BioEssays* 14, 807-815.

In various embodiments, the nucleic acids of NgR can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate
30 backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup *et al.*, (1996) *Bioorg. Med. Chem. Lett.* 4, 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in

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which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup *et al.*, (1996) above; Perry-O'Keefe *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93,14670-14675.

PNAs of NgR can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, *e.g.*, inducing transcription or translation arrest or inhibiting replication. PNAs of NgR can also be used, *e.g.*, in the analysis of single base pair mutations in a gene by, *e.g.*, PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, *e.g.*, S1 nucleases (Hyrup (1996), above); or as probes or primers for DNA sequence and hybridization (Hyrup *et al.*, (1996), above; Perry-O'Keefe (1996), above).

In another embodiment, PNAs of NgR can be modified, *e.g.*, to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of NgR can be generated that may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, *e.g.*, RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup (1996), above). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup (1996), above and Finn *et al.* (1996) *Nucleic Acids Res.* 24, 3357-3363. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry, and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl) amino-5'-deoxy-thymidine phosphoramidite, can be used between the PNA and the 5' end of DNA (Mag *et al.* (1989) *Nucleic Acids Res.* 17, 973-988). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn *et al.* (1996), above). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment

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and a 3' PNA segment. See, Petersen *et al.* (1975) *Bioorg. Med. Chem. Lett.* 5:1119-1124.

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see Letsinger *et al.*, (1989) *Proc. Natl. Acad. Sci. USA* 86, 6553-6556; Lemaitre *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 84, 648-652; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134). In addition, oligonucleotides can be modified with hybridization triggered cleavage agents (see, *e.g.*, Krol *et al.*, (1988) *Biotechniques* 6, 958-976) or intercalating agents (see, *e.g.*, Zon (1988) *Pharm. Res.* 5, 539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, a hybridization triggered cross-linking agent, a transport agent, a hybridization-triggered cleavage agent, etc.

Automated sequencing methods can be used to obtain or verify the nucleotide sequence of NgR. The NgR nucleotide sequences of the present invention are believed to be 100% accurate. However, as is known in the art, nucleotide sequence obtained by automated methods may contain some errors. Nucleotide sequences determined by automation are typically at least about 90%, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of a given nucleic acid molecule. The actual sequence may be more precisely determined using manual sequencing methods, which are well known in the art. An error in a sequence which results in an insertion or deletion of one or more nucleotides may result in a frame shift in translation such that the predicted amino acid sequence will differ from that which would be predicted from the actual nucleotide sequence of the nucleic acid molecule, starting at the point of the mutation.

Polypeptides

The invention also provides purified and isolated mammalian NgR polypeptides encoded by a polynucleotide of the invention. Presently preferred is a human NgR polypeptide comprising the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:14. Another preferred embodiment is a mouse NgR polypeptide comprising the amino acid sequence of NgR3, as set forth in SEQ ID NO:4.

One aspect of the invention pertains to isolated NgR proteins, and biologically active portions thereof, or derivatives, fragments, analogs or homologs thereof. Also provided are polypeptide fragments suitable for use as immunogens to raise anti-NgR antibodies. Preferably, fragments of NgR proteins comprise at least one biological activity of NgR. In one embodiment, native NgR proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, NgR proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a NgR protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

The invention also embraces polypeptides that have at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, at least 50% or at least 45% identity and/or homology to the preferred polypeptide of the invention. In addition, the invention embraces polypeptides having the consensus sequence shown in SEQ ID NO:6, shown in Table 5) excluding the previously characterized NgR ("NgR1"), and polypeptides comprising at least about 90% of the consensus sequence.

The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over that region of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, U, or I, in the case of nucleic acids) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the region of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The term "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 80 percent sequence identity, preferably at least 85 percent identity and often 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison region.

In one aspect, percent homology is calculated as the percentage of amino acid residues in the smaller of two sequences which align with identical amino acid residue in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to maximize alignment (Dayhoff, in ATLAS OF PROTEIN SEQUENCE AND

STRUCTURE, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972), incorporated herein by reference).

A determination of homology or identity is typically made by a computer homology program known in the art. An exemplary program is the Gap program (Wisconsin Sequence Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison, WI) using the default settings, which uses the algorithm of Smith and Waterman (*Adv. Appl. Math.*, 1981, 2, 482-489, which is incorporated herein by reference in its entirety). Employing the GAP software provided in the GCG program package, (see *Needleman and Wunsch (1970) J. Mol. Biol.* 48, 443-453) the following settings for nucleic acid sequence comparison may be used: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the analogous nucleic acid sequences referred to above exhibits a degree of identity preferably of at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99%, with the CDS (encoding) part of the DNA sequence shown in SEQ ID NOs:1, 3 or 13.

BestFit was originally written for Version 1.0 by Paul Haeberli from a careful reading of the papers by Needleman and Wunsch (1970), above, and Smith and Waterman (1981), above. The following Bestfit settings for nucleic acid sequence comparison may be used: GAP creation penalty of 8.0 and GAP extension penalty of 2, the coding region of the analogous nucleic acid sequences referred to above exhibits a degree of identity preferably of at least 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, with the CDS (encoding) part of the amino acid sequence shown in SEQ ID NOs:2, 4 or 14.

Alternatively, homology may be determined by hybridization analysis wherein a nucleic acid sequence is hybridized to the complement of a sequence encoding the aforementioned proteins under stringent, moderately stringent, or low stringent conditions. See *e.g.* Ausubel, *et al.*, (Eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, NY, 1993, and below.

Polypeptides of the invention may be isolated from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving host cells of the invention.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the NgR protein is derived, or substantially free from

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chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of NgR protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of NgR protein having less than about 30% (by dry weight) of non-NgR protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-NgR protein, still more preferably less than about 10% of non-NgR protein, and most preferably less than about 5% non-NgR protein. When the NgR protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of NgR protein in which the protein is separated from chemical precursors or other chemicals that are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of NgR protein having less than about 30% (by dry weight) of chemical precursors or non-NgR chemicals, more preferably less than about 20% chemical precursors or non-NgR chemicals, still more preferably less than about 10% chemical precursors or non-NgR chemicals, and most preferably less than about 5% chemical precursors or non-NgR chemicals.

Biologically active portions of a NgR protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the NgR protein, *e.g.*, the amino acid sequence shown in SEQ ID NO:2, 4 or 14 that include fewer amino acids than the full length NgR proteins, and exhibit at least one activity of a NgR protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the NgR protein. A biologically active portion of a NgR protein can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length.

A biologically active portion of a NgR protein of the present invention may contain at least one of the features that is conserved between the NgR proteins (*e.g.*, a conserved cysteine as the N-terminus of the mature protein, four conserved cysteines

in the N-terminus before a leucine-rich region, four conserved cysteines C-terminal with respect to a leucine repeat region, eight leucine-rich repeats, and a hydrophobic C-terminus). An alternative biologically active portion of a NgR protein may contain at least two of the above-identified domains. Another biologically active portion of a
5 NgR protein may contain at least three of the above-identified domains. Yet another biologically active portion of a NgR protein of the present invention may contain at least four of the above-identified domains.

Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one
10 or more of the functional activities of a native NgR protein.

In an embodiment, the NgR protein has an amino acid sequence shown in SEQ ID NO:2, 4 or 14. In other embodiments, the NgR protein is substantially homologous to SEQ ID NO:2, 4 or 14 and retains the functional activity of the protein of SEQ ID NO:2, 4 or 14, yet differs in amino acid sequence due to natural allelic variation or
15 mutagenesis, as described in detail below.

Accordingly, in another embodiment, the NgR protein is a protein that comprises an amino acid sequence at least about 45% homologous to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:14 and retains the functional activity of the NgR proteins of SEQ ID NO:2, 4 or 14.

20 Use of mammalian host cells is expected to provide for such post-translational modifications (*e.g.*, glycosylation, truncation, lipidation and phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Glycosylated and non-glycosylated forms of NgR polypeptides are embraced by the invention.

25 The invention also embraces variant (or analog) NgR polypeptides. In one example, insertion variants are provided wherein one or more amino acid residues supplement a NgR amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the NgR amino acid sequence. Insertional variants with additional residues at either or both termini
30 can include, for example, fusion proteins and proteins including amino acid tags or labels.

Insertion variants include NgR polypeptides wherein one or more amino acid residues are added to a NgR acid sequence or to a biologically active fragment thereof.

Variant products of the invention also include mature NgR products, *i.e.*, NgR products wherein leader or signal sequences are removed, with additional amino
5 terminal residues. The additional amino terminal residues may be derived from another protein, or may include one or more residues that are not identifiable as being derived from specific proteins. NgR products with an additional methionine residue at position -1 (Met⁻¹-NgR) are contemplated, as are variants with additional methionine and lysine residues at positions -2 and -1 (Met⁻²-Lys⁻¹-NgR). Variants of NgR with
10 additional Met, Met-Lys, Lys residues (or one or more basic residues in general) are particularly useful for enhanced recombinant protein production in bacterial host cells.

Polypeptide Variants

The invention also embraces NgR variants having additional amino acid
15 residues which result from use of specific expression systems.

As used herein, a NgR "chimeric protein" or "fusion protein" comprises a NgR polypeptide operatively linked to a non-NgR polypeptide. A "NgR polypeptide" refers to a polypeptide having an amino acid sequence corresponding to NgR, whereas a
20 "non-NgR polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein that is not homologous to the NgR protein, *e.g.*, a protein that is different from the NgR protein and that is derived from the same or a different organism. Within a NgR fusion protein the NgR polypeptide can correspond to all or a portion of a NgR protein. In one embodiment, a NgR fusion protein comprises at least one biologically active portion of a NgR protein. In another embodiment, a NgR
25 fusion protein comprises at least two biologically active portions of a NgR protein. In yet another embodiment, a NgR fusion protein comprises at least three biologically active portions of a NgR protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the NgR polypeptide and the non-NgR polypeptide are fused in-frame to each other. The non-NgR polypeptide can be fused to the N-
30 terminus or C-terminus of the NgR polypeptide.

For example, in one embodiment a NgR fusion protein comprises a NgR domain operably linked to the extracellular domain of a second protein. Such fusion

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proteins can be further utilized in screening assays for compounds which modulate NgR activity (such assays are described in detail below).

For example, use of commercially available vectors that express a desired polypeptide as part of a glutathione-S-transferase (GST) fusion product provides the
5 desired polypeptide having an additional glycine residue at position -1 after cleavage of the GST component from the desired polypeptide.

In another embodiment, the fusion protein is a NgR protein containing a heterologous signal sequence at its N-terminus. For example, the native NgR signal sequence (*i.e.*, amino acids 1-30 of SEQ ID NO:2 and amino acids 1-40 of SEQ ID
10 NO:4) can be removed and replaced with a signal sequence from another protein. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion NgR can be increased through use of a heterologous signal sequence.

In yet another embodiment, the fusion protein is a NgR-immunoglobulin fusion protein in which the NgR sequences comprising one or more domains are fused to
15 sequences derived from a member of the immunoglobulin protein family. The NgR-immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject to inhibit an interaction between NgR ligand and a NgR protein on the surface of a cell, to thereby suppress NgR-mediated signal transduction *in vivo*. NgR-immunoglobulin fusion proteins can be
20 used to affect the bioavailability of a NgR cognate ligand. Inhibition of the NgR ligand/NgR interaction may be useful therapeutically for both the treatment of proliferative and differentiative disorders, as well as modulating (*e.g.*, promoting or inhibiting) cell survival. Moreover, the NgR-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-NgR antibodies in a subject, to
25 purify NgR ligands, and in screening assays to identify molecules that inhibit the interaction of NgR with NgR ligand.

A NgR chimeric or fusion protein of the invention can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional
30 techniques, *e.g.*, by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining and

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enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, Ausubel *et al.* (Eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A NgR-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the NgR protein.

Variants resulting from expression in other vector systems are also contemplated.

Insertional variants also include fusion proteins wherein the amino terminus and/or the carboxy terminus of NgR is/are fused to another polypeptide.

In another aspect, the invention provides deletion variants wherein one or more amino acid residues in a NgR polypeptide are removed. Deletions can be effected at one or both termini of the NgR polypeptide, or with removal of one or more non-terminal amino acid residues of NgR. Deletion variants, therefore, include all fragments of a NgR polypeptide.

The invention also embraces polypeptide fragments of the sequence set forth in SEQ ID NO:2, 4 or 14 wherein the fragments maintain biological (*e.g.*, ligand binding and/or intracellular signaling) immunological properties of a NgR polypeptide. Fragments comprising at least 4, 5, 10, 15, 20, 25, 30, 35, or 40 consecutive amino acids of SEQ ID NO:2, 4 or 14 are contemplated by the invention. Preferred polypeptide fragments display antigenic properties unique to, or specific for, human NgR and its allelic and species homologs. Fragments of the invention having the desired biological and immunological properties can be prepared by any of the methods well known and routinely practiced in the art.

In still another aspect, the invention provides substitution variants of NgR polypeptides. Substitution variants include those polypeptides wherein one or more amino acid residues of a NgR polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature; however, the

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invention embraces substitutions that are also non-conservative. Conservative substitutions for this purpose may be defined as set out in Tables 2, 3, or 4 below.

Table 1.

5	X_{aa}# (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
	X ₁	G, R, M	
	X ₂	A, D, C	
	X ₃	V, T	
10	X ₄	N, P, S	
	X ₅	E, A, S	
	X ₆	nothing, K	nothing
	X ₇	V, M, P	
	X ₈	T, V	V
15	X ₉	Q, P	Q
	X ₁₀	Q, A	Q
	X ₁₁	Q, H, N	
	X ₁₂	G, N	N
	X ₁₃	L, F	F
20	X ₁₄	Q, A, S	
	X ₁₅	A, S	
	X ₁₆	V, I	
	X ₁₇	V, T, E, L	
	X ₁₈	S, G	
25	X ₁₉	L, I	
	X ₂₀	A, E, V, P	
	X ₂₁	A, S, D	
	X ₂₂	S, T	
	X ₂₃	Q, E	

$X_{aa}\#$ (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
X_{24}	IVL	
X_{25}	Q,H	Q
X_{26}	N,G	N
X_{27}	R,L	
X_{28}	T,G,R,S	
X_{29}	F,L,T,H	
X_{30}	L,V	L
X_{31}	Q,R,P	
X_{32}	Q,P,A	P
X_{33}	G,A	G
X_{34}	H,T,S	
X_{35}	S,G,R	
X_{36}	P,S,A	
X_{37}	C, nothing	nothing
X_{38}	R, nothing	nothing
X_{39}	A,N	
X_{40}	M,L	
X_{41}	V,L,T	
X_{42}	T, I	T
X_{43}	L,I	
X_{44}	Y,F,H	
X_{45}	N,V	N
X_{46}	I,L	
X_{47}	T,S,A	
X_{48}	F,Y,T,R	
X_{49}	A,H,Y,D	

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	X_{aa}# (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
	X ₅₀	P,A	P
	X ₅₁	N,S,G,A	
	X ₅₂	T,A	T
	X ₅₃	E,R,T	
5	X ₅₄	G,H	
	X ₅₅	F,L	
	X ₅₆	V,Q,H	
	X ₅₇	H,A,L	
	X ₅₈	E,Q	E
10	X ₅₉	G,S	G
	X ₆₀	R,A	R
	X ₆₁	Q,H	Q
	X ₆₂	R, H	H
	X ₆₃	T,S	
15	X ₆₄	L,V	L
	X ₆₅	A,E,D	
	X ₆₆	E,D,A	
	X ₆₇	Q,H	Q
	X ₆₈	V,E,G	
20	X ₆₉	K,R	
	X ₇₀	H,Q	
	X ₇₁	A,S,T	
	X ₇₂	Y,H	
	X ₇₃	Y,D	Y
25	X ₇₄	K,R	
	X ₇₅	G,Q	

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$X_{aa} \#$ (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
X_{76}	S,Q	S
X_{77}	A,S,E	
X_{78}	P,G	P
X_{79}	A,G,P	
X_{80}	G,N	
X_{81}	I,V,L	
X_{82}	G,R	
X_{83}	H,V,A	
X_{84}	S,A	S
X_{85}	D,E	
X_{86}	H,S,A	
X_{87}	I,L	
X_{88}	E,L,Q	
X_{89}	Y,H,A	
X_{90}	Q,P	Q
X_{91}	D, N	
X_{92}	I, L, T	
X_{93}	V,A,R	
X_{94}	V,A,G	
X_{95}	S,T	S
X_{96}	K,R	
X_{97}	L,I	L
X_{98}	W,R,S	
X_{99}	S,L	
X_{100}	L,V	L
X_{101}	G,T,P	

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	X_{aa}# (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
	X ₁₀₂	Q,P,E	
	X ₁₀₃	G,H,R	
	X ₁₀₄	I,T,V,A	
	X ₁₀₅	V,G,H	
5	X ₁₀₆	N,S	
	X ₁₀₇	E,G,Q	
	X ₁₀₈	Q,R	
	X ₁₀₉	L,V	
	X ₁₁₀	Q,A	
10	X ₁₁₁	W,G,H	
	X ₁₁₂	H,R,P	
	X ₁₁₃	K,A,H	
	X ₁₁₄	H,R	
	X ₁₁₅	D,G	
15	X ₁₁₆	H,R,S,G	
	X ₁₁₇	T,M	
	X ₁₁₈	T,I	
	X ₁₁₉	F,Y	
	X ₁₂₀	N,A	
20	X ₁₂₁	S,N	
	X ₁₂₂	T,A,S	
	X ₁₂₃	E,S,A	
	X ₁₂₄	Q,P	
	X ₁₂₅	G,T	
25	X ₁₂₆	D,E	D
	X ₁₂₇	C,A	

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	X_{aa}# (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
	X ₁₂₈	P,D	
	X ₁₂₉	V,G,P,R	
	X ₁₃₀	A,S	
	X ₁₃₁	E,Q	Q
5	X ₁₃₂	F,Y	F
	X ₁₃₃	G,A,D	
	X ₁₃₄	A,P	
	X ₁₃₅	D,A,V	
	X ₁₃₆	G,D	
10	X ₁₃₇	A,E	
	X ₁₃₈	S,P	
	X ₁₃₉	E,A	
	X ₁₄₀	L,F	
	X ₁₄₁	R,Q	
15	X ₁₄₂	R,K	R
	X ₁₄₃	R,K	R
	X ₁₄₄	F,A	
	X ₁₄₅	G,V	
	X ₁₄₆	A,D,E	
20	X ₁₄₇	T,P	
	X ₁₄₈	A,V,S	
	X ₁₄₉	T,S,L	
	X ₁₅₀	E,G,P,Q	
	X ₁₅₁	L,E,R	
25	X ₁₅₂	R,L	R
	X ₁₅₃	G, D	

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X_{aa}# (based on a NTLRRCT domain)	Column I (R1, R2, R3)	Column II (R2+R3 only)
X ₁₅₄	Q,H,A	
X ₁₅₅	Q,R	
X ₁₅₆	K,R	
X ₁₅₇	L,A,R	
X ₁₅₈	R,A	R
X ₁₅₉	V,A,E	
X ₁₆₀	E,A,N	
X ₁₆₁	F,L	F
X ₁₆₂	R,Q	
X ₁₆₃	N,A,G	

Variant polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention. Amino acids can be classified according to physical properties and contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in Table 2 (from WO 97/09433, page 10, published March 13, 1997 (PCT/GB96/02197, filed 9/6/96), immediately below.

Table 2

Conservative Substitutions I

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Aliphatic	G A P
Non-polar	I L V

Polar - uncharged	C S T M N Q
Polar - charged	D E K R
Aromatic	H F W Y
Other	N Q D E

5 Alternatively, conservative amino acids can be grouped as described in Lehninger, [BIOCHEMISTRY, Second Edition; Worth Publishers, Inc. NY, NY (1975), pp.71-77] as set out in Table 3, immediately below.

Table 3
Conservative Substitutions II

10	SIDE CHAIN CHARACTERISTIC	AMINO ACID
	Non-polar (hydrophobic)	
	A. Aliphatic:	A L I V P
	B. Aromatic:	F W
15	C. Sulfur-containing:	M
	D. Boderline:	G
	Uncharged-polar	
	A. Hydroxyl:	S T Y
	B. Amides:	N Q
	C. Sylfhydryl:	C
20	D. Boderline:	G
	Positively Charged (Basic):	K R H
	Negatively Charged (Acidic):	D E

As still another alternative, exemplary conservative substitutions are set out in
25 Table 4, below.

Table 4
Conservative Substitutions III

	Original Residue	Exemplary Substitution
30	Ala (A)	Val, Leu, Ile
	Arg (R)	Lys, Gln, Asn
	Asn (N)	Gln, His, Lys, Arg
	Asp (D)	Glu
	Cys (C)	Ser
	Gln (Q)	Asn
35	Glu (E)	Asp

	His (H)	Asn, Gln, Lys, Arg
	Ile (I)	Leu, Val, Met, Ala, Phe,
	Leu (L)	Ile, Val, Met, Ala, Phe
	Lys (K)	Arg, Gln, Asn
5	Met (M)	Leu, Phe, Ile
	Phe (F)	Leu, Val, Ile, Ala
	Pro (P)	Gly
	Ser (S)	Thr
	Thr (T)	Ser
10	Trp (W)	Tyr
	Tyr (Y)	Trp, Phe, Thr, Ser
	Val (V)	Ile, Leu, Met, Phe, Ala

In addition, amino acid residues that are conserved among family members of the NgR proteins of the present invention, as indicated by the alignment presented herein, are also predicted to be particularly unamenable to alteration. For example, NgR proteins of the present invention can contain at least one domain that is a typically conserved region in NgRs. Examples of these conserved domains include, *e.g.*, leucine-rich repeat domain. Amino acid residues that are not conserved or are only semi-conserved among members of the NgR proteins may be readily amenable to alteration.

Full-length NgRs have an LRR region characterized by the amino acid consensus sequence shown in SEQ ID NO: 19. At least some full-length NgRs also include a CT signaling (CTS) domain and a GPI domain.

The NgR domain designations used herein are defined as follows:

Domain	hNgR1 SEQ ID: 5	mNgR1 SEQ ID NO:17	hNgR2 SEQ ID: 2	hNgR3 SEQ ID: 14	mNgR3 SEQ ID: 4
Signal Seq.	1-26	1-26	1-30	—	1-40
LRRNT	27-56	27-56	31-59	—	41-69
LRR1	57-81	57-81	60-82	5-27	70-92
LRR2	82-105	82-105	83-106	28-51	93-106
LRR3	106-130	106-130	107-131	52-76	106-141
LRR4	131-154	131-154	132-155	77-100	142-165

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It should be understood that the definition of polypeptides of the invention is intended to include polypeptides bearing modifications other than insertion, deletion, or substitution of amino acid residues. By way of example, the modifications may be covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic and inorganic moieties. Such derivatives may be prepared to increase circulating half-life of a polypeptide, or may be designed to improve the targeting capacity of the polypeptide for desired cells, tissues or organs. Similarly, the invention further embraces NgR polypeptides that have been covalently modified to include one or more water-soluble polymer attachments such as polyethylene glycol, polyoxyethylene glycol or polypropylene glycol. Variants that display ligand binding properties of native NgR and are expressed at higher levels, as well as variants that provide for constitutively active receptors, are particularly useful in assays of the invention; the variants are also useful in providing cellular, tissue and animal models of diseases/conditions characterized by aberrant NgR activity.

Chemically modified NgR polypeptide compositions in which the NgR polypeptide is linked to a polymer are included within the scope of the present invention. The polymer may be water soluble to prevent precipitation of the protein in an aqueous environment, such as a physiological environment. Suitable water-soluble polymers may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxypolyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, polypropylene glycol homopolymers, a polypropylene oxide/ethylene oxide copolymer polyoxyethylated polyols (e.g. glycerol) and polyvinyl alcohol. The selected polymer is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled. Polymers may be of any molecular weight, and may be branched or unbranched, and mixtures of such polymers may also be used. When the chemically modified NgR polymer is destined for therapeutic use, pharmaceutically acceptable polymers will be selected for use.

When the polymer is to be modified by an acylation reaction, the polymer should have a single reactive ester group. Alternatively, if the polymer is to be modified by reductive alkylation, the polymer should have a single reactive aldehyde

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group. A preferred reactive aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono Cl-CIO alkoxy or aryloxy derivatives thereof (see U.S. Patent No. 5,252,714, incorporated by reference herein in its entirety).

Pegylation of NgR polypeptides may be carried out by any of the pegylation reactions known in the art, as described, for example, in the following references: *Focus on Growth Factors* 3, 4-10 (1992); EP 0 154 316; and EP 0 401 384 (each of which is incorporated by reference herein in its entirety). Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). A preferred water-soluble polymer for pegylation of polypeptides such as NgR is polyethylene glycol (PEG). As used herein, "polyethylene glycol" is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-CIO) alkoxy- or aryloxy-polyethylene glycol.

Chemical derivatization of NgR polypeptides may be performed under any suitable conditions used to react a biologically active substance with an activated polymer molecule. Methods for preparing pegylated NgR polypeptides will generally comprise the steps of (a) reacting the polypeptide with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby NgR polypeptide becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

Pegylated and other polymer:NgR polypeptides may generally be used to treat conditions that may be alleviated or modulated by administration of the NgR polypeptides described herein. However, the chemically-derivatized polymer:NgR polypeptide molecules disclosed herein may have additional activities, enhanced or reduced biological activity, or other characteristics, such as increased or decreased half-life, as compared to the nonderivatized molecules. The NgR polypeptides, fragments thereof, variants and derivatives, may be employed alone, together, or in combination with other pharmaceutical compositions. The cytokines, growth factors, antibiotics, antiinflammatories and/or chemotherapeutic agents as is appropriate for the indication being treated.

The present invention provides compositions comprising purified polypeptides of the invention. Preferred compositions comprise, in addition to the polypeptide of the invention, a pharmaceutically acceptable (*i.e.*, sterile and non-toxic) liquid, semisolid, or solid diluent that serves as a pharmaceutical vehicle, excipient or medium.

5 Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, water, saline solutions, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, glycerol, calcium phosphate, mineral oil and cocoa butter.

10 Variants that display ligand binding properties of native NgR and are expressed at higher levels, as well as variants that provide for constitutively active receptors, are particularly useful in assays of the invention; the variants are also useful in assays of the invention and in providing cellular, tissue and animal models of diseases/conditions characterized by aberrant NgR activity.

15 With the knowledge of the nucleotide sequence information disclosed in the present invention, one skilled in the art can identify and obtain nucleotide sequences which encode NgR from different sources (*i.e.*, different tissues or different organisms) through a variety of means well known to the skilled artisan and as disclosed by, for example, Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), which is
20 incorporated herein by reference in its entirety.

For example, DNA that encodes NgR may be obtained by screening of mRNA, cDNA, or genomic DNA with oligonucleotide probes generated from the NgR gene sequence information provided herein. Probes may be labeled with a detectable group, such as a fluorescent group, a radioactive atom or a chemiluminescent group in
25 accordance with procedures known to the skilled artisan and used in conventional hybridization assays, as described by, for example, Sambrook *et al.* (1989) above.

A nucleic acid molecule comprising any of the NgR nucleotide sequences described above can alternatively be synthesized by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers produced from the
30 nucleotide sequences provided herein. See U.S. Patent Nos. 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis. The PCR reaction provides a method for selectively increasing the concentration of a particular nucleic acid sequence even when that

sequence has not been previously purified and is present only in a single copy in a particular sample. The method can be used to amplify either single- or double-stranded DNA. The essence of the method involves the use of two oligonucleotide probes to serve as primers for the template-dependent, polymerase-mediated replication of a desired nucleic acid molecule.

A wide variety of alternative cloning and *in vitro* amplification methodologies are well known to those skilled in the art. Examples of these techniques are found in, for example, Berger *et al.*, *Guide to Molecular Cloning Techniques*, METHODS IN ENZYMOLOGY 152 Academic Press, San Diego, CA, which is incorporated herein by reference in its entirety.

The nucleic acid molecules of the present invention, and fragments derived therefrom, are useful for screening for restriction fragment length polymorphism (RFLP) associated with certain disorders, as well as for genetic mapping.

15 **Antibodies**

Also comprehended by the present invention are antibodies (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, bifunctional/bispecific antibodies, humanized antibodies, human antibodies, and complementary determining region (CDR)-grafted antibodies, including compounds which include CDR sequences which specifically recognize a polypeptide of the invention) specific for NgR or fragments thereof. Preferred antibodies of the invention are human antibodies which are produced and identified according to methods described in WO93/11236, published June 20, 1993, which is incorporated herein by reference in its entirety. Antibody fragments, including Fab, Fab', F(ab')₂, and F_v, are also provided by the invention. The term "specific for," when used to describe antibodies of the invention, indicates that the variable regions of the antibodies of the invention recognize and bind NgR polypeptides exclusively (*i.e.*, are able to distinguish NgR polypeptides from other known NgR polypeptides by virtue of measurable differences in binding affinity, despite the possible existence of localized sequence identity, homology, or similarity between NgR and such polypeptides).

The antigenic peptide of NgR comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2, 4 or 14 and encompasses an epitope of

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NgR such that an antibody raised against the peptide forms a specific immune complex with NgR. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of NgR that are located on the surface of the protein, *e.g.*, hydrophilic regions.

It will be understood that specific antibodies may also interact with other proteins (for example, *S. aureus* protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and, in particular, in the constant region of the molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow *et al.* in ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY (1988), Chapter 6. Antibodies that recognize and bind fragments of the NgR polypeptides of the invention are also contemplated, provided that the antibodies are specific for NgR polypeptides. Antibodies of the invention can be produced using any method well known and routinely practiced in the art.

For the production of polyclonal antibodies, various suitable host animals (*e.g.*, rabbit, goat, mouse or other mammal) may be immunized by injection with the native protein, or a synthetic variant thereof, or a derivative of the foregoing. An appropriate immunogenic preparation can contain, for example, recombinantly expressed NgR protein or a chemically synthesized NgR polypeptide. The preparation can further include an adjuvant. Various adjuvants used to increase the immunological response include, but are not limited to, Freund's (complete and incomplete), mineral gels (*e.g.*, aluminum hydroxide), surface active substances (*e.g.*, lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, etc.), human adjuvants such as *Bacille Calmette-Guerin* and *Corynebacterium parvum* or similar immunostimulatory agents. If desired, the antibody molecules directed against NgR can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction.

The term "monoclonal antibody" or "monoclonal antibody composition," as used herein, refers to a population of antibody molecules that contain only one species

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of an antigen binding site capable of immunoreacting with a particular epitope of NgR. A monoclonal antibody composition thus typically displays a single binding affinity for a particular NgR protein with which it immunoreacts. For preparation of monoclonal antibodies directed towards a particular NgR protein, or derivatives, fragments,
5 analogs or homologs thereof, any technique that provides for the production of antibody molecules by continuous cell line culture may be utilized. Such techniques include, but are not limited to, the hybridoma technique (see Kohler and Milstein (1975) *Nature* 256, 495-497); the trioma technique; the human B-cell hybridoma technique (see Kozbor *et al.*, (1983) *Immunol. Today* 4, 72) and the EBV hybridoma
10 technique to produce human monoclonal antibodies (see Cole *et al.*, (1985) in MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96). Human monoclonal antibodies may be utilized in the practice of the present invention and may be produced by using human hybridomas (see Cote *et al.*, (1983) *Proc. Natl. Acad. Sci. USA* 80, 2026-2030) or by transforming human B-cells with Epstein Barr
15 Virus *in vitro* (see Cole *et al.*, (1985), above).

According to the invention, techniques can be adapted for the production of single-chain antibodies specific to a NgR protein (see *e.g.*, U.S. Patent No. 4,946,778). In addition, methods can be adapted for the construction of Fab expression libraries (see *e.g.*, Huse *et al.*, (1989) *Science* 246, 1275-1281) to allow rapid and effective
20 identification of monoclonal F_{ab} fragments with the desired specificity for a NgR protein or derivatives, fragments, analogs or homologs thereof. Non-human antibodies can be "humanized" by techniques well known in the art. See *e.g.*, U.S. Patent No. 5,225,539. In one method, the non-human CDRs are inserted into a human antibody or consensus antibody framework sequence. Further changes can then be introduced
25 into the antibody framework to modulate affinity or immunogenicity. Antibody fragments that contain the idiotypes to a NgR protein may be produced by techniques known in the art including, but not limited to: (i) an F(ab')₂ fragment produced by pepsin digestion of an antibody molecule; (ii) an Fab fragment generated by reducing the disulfide bridges of an F(ab')₂ fragment; (iii) an Fab fragment generated by the
30 treatment of the antibody molecule with papain and a reducing agent and (iv) F_v fragments.

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In a preferred embodiment of the invention a portion of a NgR is joined to an Fc portion of an antibody to form a NgR/Fc fusion protein. Preferably, the Ig fusion protein is soluble. The NgR/Fc fusion protein may be formed by recombinant techniques as described above. In one embodiment, a portion of a NgR including the entire amino acid sequence of NgR except the C-terminal hydrophobic region is fused to an Fc portion of an antibody. In preferred embodiments, the NgR is a human NgR and the Fc is also human. More preferably, the human Fc portion is derived from an IgG antibody. In other embodiments, the N-terminal signal sequence is omitted. Such antibodies are useful in binding Nogo to prevent Nogo signaling through the NgR.

In one embodiment, methods for the screening of antibodies that possess the desired specificity include, but are not limited to, enzyme-linked immunosorbent assay (ELISA) and other immunologically-mediated techniques known within the art. In a specific embodiment, selection of antibodies that are specific to a particular domain of a NgR protein is facilitated by generation of hybridomas that bind to the fragment of a NgR protein possessing such a domain. Antibodies that are specific for one or more

domains within a NgR protein, *e.g.*, domains spanning the above-identified conserved regions of NgRs, or derivatives, fragments analogs or homologs thereof, are also provided herein.

Anti-NgR antibodies may be used in methods known within the art relating to the localization and/or quantitation of a NgR protein (*e.g.*, for use in measuring levels of the NgR protein within appropriate physiological samples, for use in diagnostic methods, for use in imaging the protein, and the like). In a given embodiment, antibodies for NgR proteins, or derivatives, fragments analogs or homologs thereof, that contain the antibody derived binding domain, are utilized as pharmacologically-active compounds [hereinafter "Therapeutics"].

An anti-NgR antibody (*e.g.*, monoclonal antibody) can be used to isolate NgR by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-NgR antibody can facilitate the purification of natural NgR from cells and of recombinantly produced NgR expressed in host cells. Moreover, an anti-NgR antibody can be used to detect NgR protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the NgR protein. Anti-NgR antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

Another aspect of the present invention is directed to methods of inducing an immune response in a mammal against a polypeptide of the invention by administering

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to the mammal an amount of the polypeptide sufficient to induce an immune response. The amount will be dependent on the animal species, size of the animal, and the like but can be determined by those skilled in the art.

Another aspect of the invention is directed to anti-idiotypic antibodies and anti-anti-idiotypic antibodies. An anti-idiotypic antibody is an antibody that recognizes determinants of another antibody (a target antibody). Generally, the anti-idiotypic antibody recognizes determinants of the antigen-binding site of the target antibody. Typically, the target antibody is a monoclonal antibody. An anti-idiotypic antibody is generally prepared by immunizing an animal (particularly, mice) of the same species and genetic type as the source of the target monoclonal antibody, with the target monoclonal antibody. The immunized animal mounts an immune response to the idiotypic determinants of the target monoclonal antibody and produces antibodies against the idiotypic determinants of the target monoclonal antibody. Antibody-producing cells, such as splenic cells, of the immunized animal may be used to generate anti-idiotypic monoclonal antibodies. Furthermore, an anti-idiotypic antibody may also be used to immunize animals to produce anti-anti-idiotypic antibodies. These immunized animals may be used to generate anti-anti-idiotypic monoclonal antibodies using standard techniques. The anti-anti-idiotypic antibodies may bind to the same epitope as the original, target monoclonal antibody used to prepare the anti-idiotypic antibody. The anti-anti-idiotypic antibodies represent other monoclonal antibodies with the same antigen specificity as the original target monoclonal antibody.

If the binding of the anti-idiotypic antibody with the target antibody is inhibited by the relevant antigen of the target antibody, and if the anti-idiotypic antibody induces an antibody response with the same specificity as the target antibody, it mimics the antigen of the target antibody. Such an anti-idiotypic antibody is an "internal image anti-idiotype" and is capable of inducing an antibody response as if it were the original antigen. (Bona and Kohler (1984) ANTI-IDIOTYPIC ANTIBODIES AND INTERNAL IMAGE, IN MONOCLONAL AND ANTI-IDIOTYPIC ANTIBODIES: PROBES FOR RECEPTOR STRUCTURE AND FUNCTION, Venter J.C. *et al.* (Eds), Alan R. Liss, New York, NY, pp 141-149, 1984). Vaccines incorporating internal image anti-idiotype antibodies have been shown to induce protective responses against viruses, bacteria, and parasites (Kennedy

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et al., (1986) 232, 220-223; 1047; McNamara *et al.*, (1985) *Science* 226, 1325-1326). Internal image anti-idiotypic antibodies have also been shown to induce immunity to tumor related antigens (Raychauhuri *et al.*, (1986) *J. Immunol.* 137, 1743-1749; Raychauhuri *et al.*, (1987) *J. Immunol.* 139, 3902-3910; Bhattacharya-Chatterjee *et al.*, (1987) *J. Immunol.* 139, 1354-1360; Bhattacharya-Chatterjee *et al.*, (1988) *J. Immunol.* 141, 1398-1403; Herlyn. *et al.* (1989) *Intern. Rev. Immunol.* 4, 347-357; Chen *et al.* (1990) *Cell Imm. Immunother. Cancer* 351-359; Herlyn *et al.*, (1991) *in vivo* 5, 615-624; Furuya *et al.* (1992) *AntiCancer Res.* 12, 27-32; Mittelman, A. *et al.* (1992) *Proc. Natl. Acad. Sci., USA* 89, 466-470; Durrant. *et al.*, (1994) *Cancer Res.* 54, 4837-4840; Mittelman. *et al.* (1994) *Cancer Res.* 54, 415-421; Schmitt. *et al.* (1994) *Hybridoma* 13, 389-396; Chakrobarty. *et al.* (1995) *J. Immunother.* 18, 95-103; Chakrobarty. *et al.* (1995) *Cancer Res.* 55, 1525-1530; Foon, K. A. *et al.* (1995) *Clin. Cancer Res.* 1, 1205-1294; Herlyn *et al.* (1995) *Hybridoma* 14, 159-166; Sclebusch *et al.* (1995) *Hybridoma* 14, 167-174; Herlyn. *et al.* (1996) *Cancer Immunol Immunother.* 43, 65-76).

Anti-idiotypic antibodies for NgR may be prepared, for example, by immunizing an animal, such as a mouse, with a immunogenic amount of a composition comprising NgR2 (SEQ ID NO:2), NgR3 (SEQ ID NOs:4 or 14), or immunogenic portion thereof, containing at least one antigenic epitope of NgR. The composition may also contain a suitable adjuvant, and any carrier necessary to provide immunogenicity. Monoclonal antibodies recognizing NgR may be prepared from the cells of the immunized animal as described above. A monoclonal antibody recognizing an epitope of NgR is then selected and used to prepare a composition comprising an immunogenic amount of the anti-NgR monoclonal antibody. Typically, a 25 to 200 µg dose of purified anti-NgR monoclonal would be sufficient in a suitable adjuvant.

Animals may be immunized 2-6 times at 14 to 30 day intervals between doses. Typically, animals are immunized by any suitable route of administration, such as intraperitoneal, subcutaneous, intravenous or a combination of these. Anti-idiotypic antibody production may be monitored during the immunization period using standard immunoassay methods. Animals with suitable titers of antibodies reactive with the target monoclonal antibodies may be reimmunized with the monoclonal antibody used as the immunogen three days before harvesting the antibody producing cells.

Preferably, spleen cells are used, although other antibody producing cells may be selected. Antibody-producing cells are harvested and fused with myeloma cells to produce *Hybridomas*, as described above, and suitable anti-idiotypic antibody-producing cells are selected.

- 5 Anti-anti-idiotypic antibodies are produced by another round of immunization and *Hybridoma* production by using the anti-idiotypic monoclonal antibody as the immunogen.

- 10 Antibodies of the invention are useful for, e.g., therapeutic purposes (by modulating activity of NgR), diagnostic purposes to detect or quantitate NgR, and purification of NgR. Therefore, kits comprising an antibody of the invention for any of the purposes described herein are also comprehended.

Kits

- 15 The present invention is also directed to kits, including pharmaceutical kits. The kits can comprise any of the nucleic acid molecules described above, any of the polypeptides described above, or any antibody which binds to a polypeptide of the invention as described above, as well appropriate controls, such as positive and/or negative controls. The kit preferably comprises additional components, such as, for example, instructions, solid support, reagents helpful for quantification, and the like.
- 20 For example, the kit can comprise: a labeled compound or agent capable of detecting NgR protein or mRNA in a biological sample; means for determining the amount of NgR in the sample; and means for comparing the amount of NgR in the sample with a standard. The compound or agent can be packaged in a suitable container.

25 Screening Assays

- 30 The DNA and amino acid sequence information provided by the present invention also makes possible identification of binding partner compounds with which a NgR polypeptide or polynucleotide will interact. Methods to identify binding partner compounds include solution assays, *in vitro* assays wherein NgR polypeptides are immobilized and cell-based assays. Identification of binding partner compounds of NgR polypeptides provides candidates for therapeutic or prophylactic intervention in pathologies associated with NgR normal and aberrant biological activity.

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The invention also provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules (*e.g.*, molecules of less than 1,000 Daltons) or other drugs) that bind to NgR proteins or have a stimulatory or inhibitory effect on, for example, NgR expression or NgR activity.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a NgR protein or polypeptide or biologically active portion thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) *Anticancer Drug Des.* 12, 145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 6909; Erb *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91, 11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37, 2678; Cho *et al.*, (1993) *Science* 261, 1303; Carrell *et al.*, (1994) *Angew Chem. Int. Ed. Engl.* 33, 2059; Carrell *et al.*, (1994) *Angew Chem. Int. Ed. Engl.* 33, 2061; and Gallop *et al.*, (1994) *J. Med. Chem.* 37, 1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *BioTechniques* 13, 412-421), or on beads (Lam (1991) *Nature* 354, 82-84), on chips (Fodor (1993) *Nature* 364, 555-556), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner, above), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89, 1865-1869) or on phage (Scott and Smith (1990) *Science* 249, 386-390; Devlin (1990) *Science* 249, 404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 6378-6382; Felici (1991) *J. Mol. Biol.* 222, 301-310; Ladner, above).

1. Cell-based Assays

The invention also provides cell-based assays to identify binding partner compounds of a NgR polypeptide. In one embodiment, the invention provides a method comprising the steps of contacting a NgR polypeptide expressed on the surface of a cell with a candidate binding partner compound and detecting binding of the candidate binding partner compound to the NgR polypeptide. In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a membrane-bound form of NgR protein, or a biologically active portion thereof, on the cell surface with a test compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the NgR protein or biologically active portion thereof.

In one embodiment, an assay is a cell-based assay in which a cell which expresses a membrane-bound form of NgR protein, or a biologically active portion thereof, on the cell surface is contacted with a test compound and the ability of the test compound to bind to a NgR protein determined. The cell, for example, can be of mammalian origin or a yeast cell. Determining the ability of the test compound to bind to the NgR protein can be accomplished, for example, by coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the NgR protein or biologically active portion thereof can be determined by detecting the labeled compound in a complex. For example, test compounds can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, test compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product. In one embodiment, the assay comprises contacting a cell which expresses a membrane-bound form of NgR protein or a biologically active portion thereof, on the cell surface with a known compound which binds NgR to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a NgR protein, wherein determining the ability of the test compound to interact with a NgR protein comprises determining the ability of the test compound to preferentially bind to NgR or a biologically active portion thereof as compared to the known compound.

Determining the ability of the test compound to modulate the activity of NgR or a biologically active portion thereof can be accomplished, for example, by determining the ability of the NgR protein to bind to or interact with a NgR target molecule. As used herein, a "target molecule" is a molecule with which a NgR protein
5 binds or interacts in nature, for example, a molecule on the surface of a cell which expresses a NgR protein, a molecule on the surface of a second cell, a molecule in the extracellular milieu, a molecule associated with the internal surface of a cell membrane or a cytoplasmic molecule. A NgR target molecule can be a non-NgR molecule or a NgR protein or polypeptide of the present invention. In one embodiment, a NgR
10 target molecule is a component of a signal transduction pathway that facilitates transduction of an extracellular signal (*e.g.*, a signal generated by binding of a compound to a membrane-bound NgR molecule) through the cell membrane and into the cell. The target, for example, can be a second intercellular protein that has catalytic activity or a protein that facilitates the association of downstream signaling
15 molecules with NgR. In a preferred embodiment, the detection comprises detecting a calcium flux or other physiological event in the cell caused by the binding of the molecule.

Specific binding molecules, including natural ligands and synthetic compounds, can be identified or developed using isolated or recombinant NgR products, NgR
20 variants, or preferably, cells expressing such products. Binding partners are useful for purifying NgR products and detection or quantification of NgR products in fluid and tissue samples using known immunological procedures. Binding molecules are also manifestly useful in modulating (*i.e.*, blocking, inhibiting or stimulating) biological activities of NgR, especially those activities involved in signal transduction.

25

2. Cell-free Assays

(a) Direct binding:

The invention includes several assay systems for identifying NgR binding partners. In solution assays, methods of the invention comprise the steps of (a)
30 contacting a NgR polypeptide with one or more candidate binding partner compounds and (b) identifying the compounds that bind to the NgR polypeptide. Identification of the compounds that bind the NgR polypeptide can be achieved by isolating the NgR

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polypeptide/binding partner complex and separating the binding partner compound from the NgR polypeptide. An additional step of characterizing the physical, biological and/or biochemical properties of the binding partner compound is also comprehended in another embodiment of the invention. In one aspect, the NgR polypeptide/binding partner complex is isolated using an antibody immunospecific for either the NgR polypeptide or the candidate binding partner compound.

In still other embodiments, either the NgR polypeptide or the candidate binding partner compound comprises a label or tag that facilitates its isolation, and methods of the invention to identify binding partner compounds include a step of isolating the NgR polypeptide/binding partner complex through interaction with the label or tag. An exemplary tag of this type is a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAG[®] tag (Eastman Kodak, Rochester, NY), well known and routinely used in the art, are embraced by the invention.

(b) Immobilized NgR

In one variation of an *in vitro* assay, the invention provides a method comprising the steps of (a) contacting an immobilized NgR polypeptide, or a biologically active fragment thereof with a candidate binding partner compound and (b) detecting binding of the candidate compound to the NgR polypeptide. In an alternative embodiment, the candidate binding partner compound is immobilized and binding of NgR is detected. Immobilization is accomplished using any of the methods well known in the art, including covalent bonding to a support, a bead or a chromatographic resin, as well as non-covalent, high affinity interactions such as antibody binding, or use of streptavidin/biotin binding wherein the immobilized compound includes a biotin moiety. Binding of a test compound to NgR, or interaction of NgR with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided that adds a domain that allows one or both of the proteins to be bound to a matrix. For example, and not by way of limitation, GST-NgR fusion proteins or GST-target fusion proteins can be

adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, that are then combined with the test compound or the test compound and either the non-adsorbed target protein or NgR protein, and the mixture is incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, and the complexes determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of NgR binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either NgR or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated NgR or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with NgR or target molecules, but which do not interfere with binding of the NgR protein to its target molecule, can be derivatized to the wells of the plate, and unbound target or NgR trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the NgR or target molecule, as well as enzyme-linked assays that rely on detecting an enzymatic activity associated with the NgR or target molecule.

Detection of binding can be accomplished (i) using a radioactive label on the compound that is not immobilized, (ii) using of a fluorescent label on the non-immobilized compound, (iii) using an antibody immunospecific for the non-immobilized compound, (iv) using a label on the non-immobilized compound that excites a fluorescent support to which the immobilized compound is attached, (v) determining the activity of the NgR, as well as other techniques well known and routinely practiced in the art.

Determining the activity of the target molecule, for example, may be accomplished by detecting induction of a cellular second messenger of the target (*i.e.* intracellular Ca^{2+} , diacylglycerol, IP_3 , etc.), detecting catalytic/enzymatic activity of the target on an appropriate substrate, detecting the induction of a reporter gene (comprising
5 a NgR-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, cell survival, cellular differentiation, or cell proliferation.

(c) Competition experiments

10 In yet another embodiment, the assay comprises contacting the NgR protein or biologically active portion thereof with a known compound which binds NgR to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a NgR protein, wherein determining the ability of the test compound to interact with a NgR protein comprises determining
15 the ability of the test compound to preferentially bind to NgR or biologically active portion thereof as compared to the known compound.

In yet another embodiment, the cell-free assay comprises contacting the NgR protein or biologically active portion thereof with a known compound which binds NgR to form an assay mixture, contacting the assay mixture with a test compound, and
20 determining the ability of the test compound to interact with a NgR protein, wherein determining the ability of the test compound to interact with a NgR protein comprises determining the ability of the NgR protein to modulate the activity of a NgR target molecule.

The cell-free assays of the present invention are amenable to use of both the
25 soluble form or the membrane-bound form of NgR. In the case of cell-free assays comprising the membrane-bound form of NgR, it may be desirable to utilize a solubilizing agent such that the membrane-bound form of NgR is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide,
30 decanoyl-N-methylglucamide, Triton[®] X-100, Triton[®] X-114, Thesit[®], Isotridecypoly(ethylene glycol ether)_n, 3-(3-cholamidopropyl)dimethylamminiol-1-propane sulfonate (CHAPS), 3-(3-cholamidopropyl)dimethylamminiol-2-hydroxy-1-

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propane sulfonate (CHAPSO), or N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate.

Modulators

5 Agents that modulate (*i.e.*, increase, decrease, or block) NgR activity or expression may be identified by incubating a putative modulator with a cell containing a NgR polypeptide or polynucleotide and determining the effect of the putative modulator on NgR activity or expression. The selectivity of a compound that modulates the activity of NgR can be evaluated by comparing its effects on NgR to its effect on other NgR compounds. Selective modulators may include, for example, antibodies and other proteins, peptides or organic molecules which specifically bind to a NgR polypeptide or a NgR-encoding nucleic acid. Modulators of NgR activity will be therapeutically useful in treatment of diseases and physiological conditions in which normal or aberrant NgR activity is involved. NgR polynucleotides, polypeptides and modulators may be used in the treatment of such diseases and conditions associated with demyelination. NgR polynucleotides and polypeptides, as well as NgR modulators, may also be used in diagnostic assays for such diseases or conditions.

Methods of the invention to identify modulators include variations on any of the methods described above to identify binding partner compounds, the variations including techniques wherein a binding partner compound has been identified and the binding assay is carried out in the presence and absence of a candidate modulator. A modulator is identified in those instances where binding between the NgR polypeptide and the binding partner compound changes in the presence of the candidate modulator compared to binding in the absence of the candidate modulator compound. A modulator that increases binding between the NgR polypeptide and the binding partner compound is described as an enhancer or activator, and a modulator that decreases binding between the NgR polypeptide and the binding partner compound is described as an inhibitor.

In another embodiment, modulators of NgR expression may be identified in a method wherein a cell is contacted with a candidate compound and the expression of NgR mRNA or protein in the cell is determined. The level of expression of NgR mRNA or protein in the presence of the candidate compound is compared to the level

of expression of NgR mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of NgR expression based on this comparison. For example, when expression of NgR mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of NgR mRNA or protein expression. Alternatively, when expression of NgR mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of NgR mRNA or protein expression. The level of NgR mRNA or protein expression in the cells can be determined by methods described herein for detecting NgR mRNA or protein.

High Throughput Screening

The invention also comprehends high-throughput screening (HTS) assays to identify compounds that interact with or inhibit biological activity (*i.e.*, affect enzymatic activity, binding activity, etc.) of a NgR polypeptide. HTS assays permit screening of large numbers of compounds in an efficient manner. Cell-based HTS systems are contemplated to investigate NgR receptor-ligand interaction. HTS assays are designed to identify "hits" or "lead compounds" having the desired property, from which modifications can be designed to improve the desired property. Chemical modification of the "hit" or "lead compound" is often based on an identifiable structure/activity relationship between the "hit" and the NgR polypeptide.

Another aspect of the present invention is directed to methods of identifying compounds that bind to either NgR or nucleic acid molecules encoding NgR, comprising contacting NgR, or a nucleic acid molecule encoding the same, with a compound, and determining whether the compound binds NgR or a nucleic acid molecule encoding the same. Binding can be determined by binding assays which are well known to the skilled artisan, including, but not limited to, gel-shift assays, Western blots, radiolabeled competition assay, phage-based expression cloning, co-fractionation by chromatography, co-precipitation, cross linking, interaction trap/two-hybrid analysis, southwestern analysis, ELISA, and the like, which are described in, for example, Ausubel *et al.* (Eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, 1999, John Wiley & Sons, NY, which is incorporated herein by reference in

its entirety. The NgR proteins, for example, can be used as "bait proteins" in a two-hybrid assay or three hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos *et al.*, (1993) *Cell* 72, 223-232; Madura *et al.*, (1993) *J. Biol. Chem.* 268, 12046-12054; Bartel *et al.*, (1993) *BioTechniques* 14, 920-924; Iwabuchi *et al.*, (1993) *Oncogene* 8, 1693-1696; and Brent WO 94/10300), to identify other proteins that bind to or interact with NgR ("NgR-binding proteins" or "NgR-bp") and modulate NgR activity. Such NgR-binding proteins are also likely to be involved in the propagation of signals by the NgR proteins as, for example, upstream or downstream elements of the NgR pathway.

Other assays may be used to identify specific ligands of a NgR receptor, including assays that identify ligands of the target protein through measuring direct binding of test ligands to the target protein, as well as assays that identify ligands of target proteins through affinity ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Alternatively, such binding interactions are evaluated indirectly using the yeast two-hybrid system described in Fields *et al.*, (1989) *Nature* 340, 245-246, and Fields *et al.*, (1994) *Trends Genet.* 10, 286-292, both of which are incorporated herein by reference. The two-hybrid system is a genetic assay based on the modular nature of most transcription factors used for detecting interactions between two proteins or polypeptides. It can be used to identify proteins that bind to a known protein of interest, or to delineate domains or residues critical for an interaction. Variations on this methodology have been developed to clone genes that encode DNA binding proteins, to identify peptides that bind to a protein, and to screen for drugs. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA binding domain that binds to an upstream activation sequence (UAS) of a reporter gene, and is generally performed in yeast. The assay requires the construction of two hybrid genes encoding (1) a DNA-binding domain that is fused to a first protein and (2) an activation domain fused to a second protein. The DNA-binding domain targets the first hybrid protein to the UAS of the reporter gene; however, because most proteins lack an activation domain, this DNA-binding hybrid protein does not activate transcription of the reporter gene. The second hybrid protein, which contains the activation domain, cannot by itself activate expression of the reporter gene because it does not bind the UAS. However, when both hybrid proteins are present, the

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noncovalent interaction of the first and second proteins tethers the activation domain to the UAS, activating transcription of the reporter gene. For example, when the first protein is a NgR gene product, or fragment thereof, that is known to interact with another protein or nucleic acid, this assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene is monitored as different test agents are added to the system. The presence of an inhibitory agent results in lack of a reporter signal. The compounds to be screened include (which may include compounds that are suspected to bind NgR, or a nucleic acid molecule encoding the same), but are not limited to, extracellular, intracellular, biological or chemical origin.

The function of the NgR gene product is unclear and no ligands have yet been found which bind the gene product. The yeast two-hybrid assay is useful to identify proteins that bind to the gene product. In an assay to identify proteins that bind to a NgR receptor, or fragment thereof, a fusion polynucleotide encoding both a NgR receptor (or fragment) and a UAS binding domain (*i.e.*, a first protein) may be used. In addition, a large number of hybrid genes each encoding a different second protein fused to an activation domain are produced and screened in the assay. Typically, the second protein is encoded by one or more members of a total cDNA or genomic DNA fusion library, with each second protein-coding region being fused to the activation domain. This system is applicable to a wide variety of proteins, and it is not even necessary to know the identity or function of the second binding protein. The system is highly sensitive and can detect interactions not revealed by other methods; even transient interactions may trigger transcription to produce a stable mRNA that can be repeatedly translated to yield the reporter protein.

Other assays may be used to search for agents that bind to the target protein. One such screening method to identify direct binding of test ligands to a target protein is described in U.S. Patent No. 5,585,277, incorporated herein by reference. This method relies on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a test ligand binds to the folded form of a target protein (*i.e.*, when the test ligand is a ligand of the target protein), the target protein molecule bound by the ligand remains in its folded state. Thus, the folded target protein is present to a greater extent in the presence of a test ligand which binds the target protein, than in the absence of a ligand. Binding of

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the ligand to the target protein can be determined by any method which distinguishes between the folded and unfolded states of the target protein. The function of the target protein need not be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a test ligand, including, but not limited to, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

Another method for identifying ligands of a target protein is described in Wieboldt *et al.* (1997) *Anal. Chem.* 69:1683-1691, incorporated herein by reference. This technique screens combinatorial libraries of 20-30 agents at a time in solution phase for binding to the target protein. Agents that bind to the target protein are separated from other library components by simple membrane washing. The specifically selected molecules that are retained on the filter are subsequently liberated from the target protein and analyzed by HPLC and pneumatically assisted electrospray (ion spray) ionization mass spectroscopy. This procedure selects library components with the greatest affinity for the target protein, and is particularly useful for small molecule libraries.

The methods of the invention also embrace ligands, especially neuropeptides, that are attached to a label, such as a radiolabel (*e.g.*, ^{125}I , ^{35}S , ^{32}P , ^{33}P , ^3H), a fluorescence label, a chemiluminescent label, an enzymic label and an immunogenic label. Modulators falling within the scope of the invention include, but are not limited to, non-peptide molecules such as non-peptide mimetics, non-peptide allosteric effectors, and peptides. The NgR polypeptide or polynucleotide employed in such a test may either be free in solution, attached to a solid support, borne on a cell surface or located intracellularly or associated with a portion of a cell. One skilled in the art can, for example, measure the formation of complexes between NgR and the compound being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between NgR and its substrate caused by the compound being tested.

Another aspect of the present invention is directed to methods of identifying compounds which modulate (*i.e.*, increase or decrease) activity of NgR comprising contacting NgR with a compound, and determining whether the compound modifies activity of NgR. The activity in the presence of the test compared is measured to the

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activity in the absence of the test compound. Where the activity of the sample containing the test compound is higher than the activity in the sample lacking the test compound, the compound will have increased activity. Similarly, where the activity of the sample containing the test compound is lower than the activity in the sample lacking the test compound, the compound will have inhibited activity.

The present invention is particularly useful for screening compounds by using NgR in any of a variety of drug screening techniques. The compounds to be screened include (which may include compounds which are suspected to modulate NgR activity), but are not limited to, extracellular, intracellular, biologic or chemical origin. The NgR polypeptide employed in such a test may be in any form, preferably, free in solution, attached to a solid support, borne on a cell surface or located intracellularly. One skilled in the art can, for example, measure the formation of complexes between NgR and the compound being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between Nogo-R and its substrate caused by the compound being tested.

The activity of NgR polypeptides of the invention can be determined by, for example, examining the ability to bind or be activated by chemically synthesized peptide ligands. Alternatively, the activity of the NgR can be assayed by examining their ability to bind calcium ions, hormones, chemokines, neuropeptides, neurotransmitters, nucleotides, lipids, odorants and photons. Alternatively, the activity of the NgR can be determined by examining the activity of effector molecules including, but not limited to, adenylate cyclase, phospholipases and ion channels. Thus, modulators of NgR activity may alter a NgR receptor function, such as a binding property of a receptor or an activity. In various embodiments of the method, the assay may take the form of an ion flux assay, a yeast growth assay, a non-hydrolyzable GTP assay such as a [^{35}S]-GTP S assay, a cAMP assay, an inositol triphosphate assay, a diacylglycerol assay, an Aequorin assay, a Luciferase assay, a FLIPR assay for intracellular Ca^{2+} concentration, a mitogenesis assay, a MAP Kinase activity assay, an arachidonic acid release assay (*e.g.*, using [^3H]-arachidonic acid) and an assay for extracellular acidification rates, as well as other binding or function-based assays of NgR activity that are generally known in the art. NgR activity can be determined by methodologies that are used to assay for FaRP activity, which is well known to those

skilled in the art. Biological activities of NgR receptors according to the invention include, but are not limited to, the binding of a natural or an unnatural ligand, as well as any one of the functional activities of NgRs known in the art. Non-limiting examples of NgR activities include transmembrane signaling of various forms, which may involve phosphatidylinositol (PI) association and/or the exertion of an influence over PI; another exemplary activity of NgRs is the binding of accessory proteins or polypeptides that differ from known GPI proteins.

The modulators of the invention exhibit a variety of chemical structures, which can be generally grouped into non-peptide mimetics of natural NgR receptor ligands, peptide and non-peptide allosteric effectors of NgR receptors, and peptides that may function as activators or inhibitors (competitive, uncompetitive and non-competitive) (e.g., antibody products) of NgR receptors. The invention does not restrict the sources for suitable modulators, which may be obtained from natural sources such as plant, animal or mineral extracts, or non-natural sources such as small molecule libraries, including the products of combinatorial chemical approaches to library construction, and peptide libraries.

Other assays can be used to examine enzymatic activity including, but not limited to, photometric, radiometric, HPLC, electrochemical, and the like, which are described in, for example, ENZYME ASSAYS: A PRACTICAL APPROACH, Eisenthal and Danson (Eds.), 1992, Oxford University Press, which is incorporated herein by reference in its entirety.

The use of cDNAs in drug discovery programs is well-known; assays capable of testing thousands of unknown compounds per day in high-throughput screens (HTSs) are thoroughly documented. The literature is replete with examples of the use of radiolabelled ligands in HTS binding assays for drug discovery (see Williams (1991) *Med. Res. Rev.*, 11, 147-184; Sweetnam *et al.*, (1993) *J. Nat. Prod.* 56, 441-455 for review). Recombinant receptors are preferred for binding assay HTS because they allow for better specificity (higher relative purity), provide the ability to generate large amounts of receptor material, and can be used in a broad variety of formats (see Hodgson (1992) *Bio/Technology* 10, 973-980; each of which is incorporated herein by reference in its entirety).

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A variety of heterologous systems is available for functional expression of recombinant receptors that are well known to those skilled in the art. Such systems include bacteria (Strosberg *et al.* (1992) *Trends Pharmacol. Sci.* 13, 95-98), yeast (Pausch (1997) *Trends Biotechnol.* 15, 487-494), several kinds of insect cells (Vanden
5 Broeck (1996) *Int. Rev. Cytol.* 164, 189-268), amphibian cells (Jayawickreme *et al.* (1997) *Curr. Opin. Biotechnol.* 8, 629-634) and several mammalian cell lines (CHO, HEK293, COS, etc.; see Gerhardt *et al.* (1997) *Eur. J. Pharmacol.* 334, 1-23). These examples do not preclude the use of other possible cell expression systems, including cell lines obtained from nematodes (PCT application WO 98/37177).

10 In preferred embodiments of the invention, methods of screening for compounds which modulate NgR activity comprise contacting test compounds with NgR and assaying for the presence of a complex between the compound and NgR. In such assays, the ligand is typically labeled. After suitable incubation, free ligand is separated from that present in bound form, and the amount of free or uncomplexed
15 label is a measure of the ability of the particular compound to bind to NgR.

In another embodiment of the invention, high throughput screening for compounds having suitable binding affinity to NgR is employed. Briefly, large numbers of different small peptide test compounds are synthesized on a solid substrate. The peptide test compounds are contacted with NgR and washed. Bound NgR is then
20 detected by methods well known in the art. Purified polypeptides of the invention can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the protein and immobilize it on the solid support.

Generally, an expressed NgR can be used for HTS binding assays in
25 conjunction with its defined ligand. The identified peptide is labeled with a suitable radioisotope, including, but not limited to, ^{125}I , ^3H , ^{35}S or ^{32}P , by methods that are well known to those skilled in the art. Alternatively, the peptides may be labeled by well-known methods with a suitable fluorescent derivative (Baindur *et al.* (1994) *Drug Dev. Res.* 33, 373-398; Rogers (1997) *Drug Discov. Today* 2, 156-160). Radioactive
30 ligand specifically bound to the receptor in membrane preparations made from the cell line expressing the recombinant protein can be detected in HTS assays in one of several standard ways, including filtration of the receptor-ligand complex to separate

- bound ligand from unbound ligand (Williams (1991) *Med. Res. Rev.* 11, 147-184; Sweetnam *et al.* (1993) *J. Nat. Prod.* 56, 441-455). Alternative methods include a scintillation proximity assay (SPA) or a FlashPlate format in which such separation is unnecessary (Nakayama (1998) *Curr. Opin. Drug Disc. Dev.* 1, 85-91 Bossé *et al.* (1998) *J. Biomol. Screening* 3, 285-292). Binding of fluorescent ligands can be detected in various ways, including fluorescence energy transfer (FRET), direct spectrophotofluorometric analysis of bound ligand, or fluorescence polarization (Rogers (1997) *Drug Discov. Today* 2, 156-160; Hill (1998) *Curr. Opin. Drug Disc. Dev.* 1, 92-97).
- 10 Examples of such biological responses include, but are not limited to, the following: the ability to survive in the absence of a limiting nutrient in specifically engineered yeast cells (Pausch (1997) *Trends in Biotechnol.* 15, 487-494); changes in intracellular Ca^{2+} concentration as measured by fluorescent dyes (Murphy *et al.* (1998) *Cur. Opin. Drug Disc. Dev.* 1, 192-199). Fluorescence changes can also be used to
- 15 monitor ligand-induced changes in membrane potential or intracellular pH; an automated system suitable for HTS has been described for these purposes (Schroeder *et al.* (1996) *J. Biomol. Screening* 1, 75-80). Melanophores prepared from *Xenopus laevis* show a ligand-dependent change in pigment organization in response to heterologous NgR activation; this response is adaptable to HTS formats
- 20 (Jayawickreme *et al.* (1997) *Curr. Opin. Biotechnol.* 8, 629-634). Assays are also available for the measurement of common second messengers, including cAMP, phosphoinositides and arachidonic acid, but these are not generally preferred for HTS.
- Preferred methods of HTS employing these receptors include permanently transfected CHO cells, in which agonists and antagonists can be identified by the ability
- 25 to transduce the signal for the binding of Nogo in membranes prepared from these cells through the putative GPI anchor. In another embodiment of the invention, permanently transfected CHO cells could be used for the preparation of membranes which contain significant amounts of the recombinant receptor proteins; these membrane preparations would then be used in receptor binding assays, employing the
- 30 radiolabelled ligand specific for the particular receptor. Alternatively, a functional assay, such as fluorescent monitoring of ligand-induced changes in internal Ca^{2+} concentration or membrane potential in permanently transfected CHO cells containing

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each of these receptors individually or in combination would be preferred for HTS. Equally preferred would be an alternative type of mammalian cell, such as HEK293 or COS cells, in similar formats. More preferred would be permanently transfected insect cell lines, such as *Drosophila* S2 cells. Even more preferred would be recombinant yeast cells expressing the *Drosophila melanogaster* receptors in HTS formats well known to those skilled in the art (e.g., Pausch (1997), above).

The invention contemplates a multitude of assays to screen and identify inhibitors of ligand binding to NgR receptors. In one example, the NgR receptor is immobilized and interaction with a binding partner is assessed in the presence and absence of a candidate modulator such as an inhibitor compound. In another example, interaction between the NgR receptor and its binding partner is assessed in a solution assay, both in the presence and absence of a candidate inhibitor compound. In either assay, an inhibitor is identified as a compound that decreases binding between the NgR receptor and its binding partner. Another contemplated assay involves a variation of the di-hybrid assay wherein an inhibitor of protein/protein interactions is identified by detection of a positive signal in a transformed or transfected host cell, as described in PCT publication number WO 95/20652, published August 3, 1995.

Candidate modulators contemplated by the invention include compounds selected from libraries of either potential activators or potential inhibitors. There are a number of different libraries used for the identification of small molecule modulators, including: (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules. Chemical libraries consist of random chemical structures, some of which are analogs of known compounds or analogs of compounds that have been identified as "hits" or "leads" in other drug discovery screens, some of which are derived from natural products, and some of which arise from non-directed synthetic organic chemistry. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms that are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see Cane *et al.*, *Science* (1998) 282, 63-68. Combinatorial libraries are composed of

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large numbers of peptides, oligonucleotides, or organic compounds as a mixture. These libraries are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning, or proprietary synthetic methods. Of particular interest are non-peptide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see Myers (1997) *Curr. Opin. Biotechnol.* 8, 701-707. Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

Still other candidate inhibitors contemplated by the invention can be designed and include soluble forms of binding partners, as well as such binding partners as chimeric, or fusion, proteins. A "binding partner" as used herein broadly encompasses non-peptide modulators, as well as such peptide modulators as neuropeptides other than natural ligands, antibodies, antibody fragments, and modified compounds comprising antibody domains that are immunospecific for the expression product of the identified NgR gene.

Other embodiments of the invention comprise using competitive screening assays in which neutralizing antibodies capable of binding a polypeptide of the invention specifically compete with a test compound for binding to the polypeptide. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants with NgR. Radiolabeled competitive binding studies are described in Lin *et al.*, (1997) *Antimicrob. Agents Chemother.* 41, 2127-2131, the disclosure of which is incorporated herein by reference in its entirety.

In other embodiments of the invention, the polypeptides of the invention are employed as a research tool for identification, characterization and purification of interacting, regulatory proteins. Appropriate labels are incorporated into the polypeptides of the invention by various methods known in the art and the polypeptides are used to capture interacting molecules. For example, molecules are incubated with the labeled polypeptides, washed to remove unbound polypeptides, and the polypeptide complex is quantified. Data obtained using different concentrations of

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polypeptide are used to calculate values for the number, affinity, and association of polypeptide with the protein complex.

Labeled polypeptides are also useful as reagents for the purification of molecules with which the polypeptide interacts including, but not limited to, inhibitors.

- 5 In one embodiment of affinity purification, a polypeptide is covalently coupled to a chromatography column. Cells and their membranes are extracted, and various cellular subcomponents are passed over the column. Molecules bind to the column by virtue of their affinity to the polypeptide. The polypeptide-complex is recovered from the column, dissociated and the recovered molecule is subjected to protein sequencing.
- 10 This amino acid sequence is then used to identify the captured molecule or to design degenerate oligonucleotides for cloning the corresponding gene from an appropriate cDNA library.

- Alternatively, compounds may be identified which exhibit similar properties to the ligand for the NgR of the invention, but which are smaller and exhibit a longer half
- 15 time than the endogenous ligand in a human or animal body. When an organic compound is designed, a molecule according to the invention is used as a "lead" compound. The design of mimetics to known pharmaceutically active compounds is a well-known approach in the development of pharmaceuticals based on such "lead" compounds. Mimetic design, synthesis and testing are generally used to avoid
- 20 randomly screening a large number of molecules for a target property. Furthermore, structural data deriving from the analysis of the deduced amino acid sequences encoded by the DNAs of the present invention are useful to design new drugs, more specific and therefore with a higher pharmacological potency.

- Comparison of the protein sequence of the present invention with the
- 25 sequences present in all the available databases showed a significant homology with the transmembrane portion of G protein coupled receptors. Accordingly, computer modeling can be used to develop a putative tertiary structure of the proteins of the invention based on the available information of the transmembrane domain of other proteins. Thus, novel ligands based on the predicted structure of NgR can be
- 30 designed.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

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Compositions and Pharmaceutical Compositions

In a particular embodiment, the novel molecules identified by the screening methods according to the invention are low molecular weight organic molecules, in which case a composition or pharmaceutical composition can be prepared thereof for oral or parenteral administration. The compositions, or pharmaceutical compositions, comprising the nucleic acid molecules, vectors, polypeptides, antibodies and compounds identified by the screening methods described herein, typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The nature of the carrier or other ingredients will depend on the specific route of administration and particular embodiment of the invention to be administered. Examples of techniques and protocols that are useful in this context are, *inter alia*, found in Remington's PHARMACEUTICAL SCIENCES, 16th ed., (1980) Osol, A (Ed.), which is incorporated herein by reference in its entirety. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solution, dextrose solution and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include oral and parenteral (*e.g.*, intravenous, intradermal, subcutaneous, inhalation, transdermal (topical), transmucosal and rectal administration). Solutions or suspensions used for parenteral, intradermal or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic

acid; buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a NgR protein or anti-NgR antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of

preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They
5 can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed.
10 Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel or corn starch; a lubricant
15 such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable
20 propellant, *e.g.*, a gas such as carbon dioxide or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means.
For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile
25 salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with
30 conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

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In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811. It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by any of a number of routes, *e.g.*, as described in U.S. Patent No. 5,703,055. Delivery can thus also include, *e.g.*, intravenous injection, local administration (see U.S. Patent No. 5,328,470) or stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91, 3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack or dispenser together with instructions for administration.

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The dosage of these low molecular weight compounds will depend on the disease state or condition to be treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating human or animals, between approximately 0.5 mg/kg of body weight to 500 mg/kg of body weight of the compound can be administered. Therapy is typically administered at lower dosages and is continued until the desired therapeutic outcome is observed.

Another aspect of the present invention is the use of the NgR nucleotide sequences disclosed herein for identifying homologs of the Nogo-R, in other animals, including but not limited to humans and other mammals and invertebrates. Any of the nucleotide sequences disclosed herein, or any portion thereof, can be used, for example, as probes to screen databases or nucleic acid libraries, such as, for example, genomic or cDNA libraries, to identify homologs using screening procedures well known to those skilled in the art. Accordingly, homologs having at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and most preferably at least 100% homology with NgR sequences can be identified.

The present compounds and methods, including nucleic acid molecules, polypeptides, antibodies, compounds identified by the screening methods described herein, have a variety of pharmaceutical applications and may be used, for example, to treat or prevent unregulated cellular growth, such as cancer cell and tumor growth. In a particular embodiment, the present molecules are used in gene therapy. For a review of gene therapy procedures, see *e.g.* Anderson *Science* (1992) 256, 808-813, which is incorporated herein by reference in its entirety.

The present invention also encompasses a method of agonizing (stimulating) or antagonizing a NgR natural binding partner associated activity in a mammal comprising administering to said mammal an agonist or antagonist to one of the above disclosed polypeptides in an amount sufficient to effect said agonism or antagonism. One embodiment of the present invention, then, is a method of treating diseases in a mammal with an agonist or antagonist of the protein of the present invention comprising administering the agonist or antagonist to a mammal in an amount sufficient to agonize or antagonize NgR-associated functions.

Methods of determining the dosages of compounds to be administered to a patient and modes of administering compounds to an organism are disclosed in U.S. Application Serial No. 08/702,282, filed August 23, 1996, and International patent publication number WO 96/22976, published August 1, 1996, both of which are
5 incorporated herein by reference in their entirety, including any drawings, figures or tables. Those skilled in the art will appreciate that such descriptions are applicable to the present invention and can be easily adapted to it.

The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used and the size and physiological condition
10 of the patient. Therapeutically effective doses for the compounds described herein can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that initially takes into account the IC_{50} as determined in cell culture assays. The animal model data can be used to more accurately determine useful doses in humans.

15 Plasma half-life and biodistribution of the drug and metabolites in the plasma, tumors and major organs can also be determined to facilitate the selection of drugs most appropriate to inhibit a disorder. Such measurements can be carried out. For example, HPLC analysis can be performed on the plasma of animals treated with the drug and the location of radiolabeled compounds can be determined using detection
20 methods such as X-ray, CAT scan and MRI. Compounds that show potent inhibitory activity in the screening assays, but have poor pharmacokinetic characteristics, can be optimized by altering the chemical structure and retesting. In this regard, compounds displaying good pharmacokinetic characteristics can be used as a model.

Toxicity studies can also be carried out by measuring the blood cell
25 composition. For example, toxicity studies can be carried out in a suitable animal model as follows: (1) the compound is administered to mice (an untreated control mouse should also be used); (2) blood samples are periodically obtained via the tail vein from one mouse in each treatment group; and (3) the samples are analyzed for red and white blood cell counts, blood cell composition and the percent of lymphocytes
30 versus polymorphonuclear cells. A comparison of results for each dosing regime with the controls indicates if toxicity is present.

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At the termination of each toxicity study, further studies can be carried out by sacrificing the animals (preferably, in accordance with the American Veterinary Medical Association guidelines Report of the American Veterinary Medical Assoc. Panel on Euthanasia, (1993) *J. Am. Vet. Med. Assoc.* 202:229-249). Representative
5 animals from each treatment group can then be examined by gross necropsy for immediate evidence of metastasis, unusual illness or toxicity. Gross abnormalities in tissue are noted and tissues are examined histologically. Compounds causing a reduction in body weight or blood components are less preferred, as are compounds having an adverse effect on major organs. In general, the greater the adverse effect the
10 less preferred the compound.

For the treatment of cancers the expected daily dose of a hydrophobic pharmaceutical agent is between 1 to 500 mg/day, preferably 1 to 250 mg/day, and most preferably 1 to 50 mg/day. Drugs can be delivered less frequently provided plasma levels of the active moiety are sufficient to maintain therapeutic effectiveness.
15 Plasma levels should reflect the potency of the drug. Generally, the more potent the compound the lower the plasma levels necessary to achieve efficacy.

NgR mRNA transcripts have been found in the brain and heart. SEQ ID NOs: 1 and/or, 3 will, as detailed above, enable screening the endogenous neurotransmitters/hormones/ligands which activate, agonize, or antagonize NgR and
20 for compounds with potential utility in treating disorders including CNS disorders (e.g., stroke) and degenerative disorders such as those associated with demyelination.

For example, NgR receptor activation may mediate the prevention of neurite outgrowth. Inhibition would be beneficial in both chronic and acute brain injury. See, e.g., Donovan *et al.*, (1997) *J. Neurosci.* 17, 5316-5326; Turgeon *et al.*, (1998) *J. Neurosci.* 18, 6882-6891; Smith-Swintosky *et al.*, (1997) *J. Neurochem.* 69,
25 1890-1896; Gill *et al.*, (1998) *Brain Res.* 797, 321-327; Suidan *et al.*, (1996) *Semin. Thromb. Hemost.* 22, 125-133.

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Pharmacogenomics

Agents, or modulators that have a stimulatory or inhibitory effect on NgR activity (*e.g.*, NgR gene expression), as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (*e.g.*, a disease condition such as a demyelination disorder) associated with aberrant NgR activity. In conjunction with such treatment, the pharmacogenomics (*i.e.*, the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered.

Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permits the selection of effective agents (*e.g.*, drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens.

Accordingly, the activity of NgR protein, expression of NgR nucleic acid or mutation content of NgR genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See *e.g.*, Eichelbaum (1996) *Clin. Exp. Pharmacol. Physiol.* 23, 983-985 and Linder (1997) *Clin. Chem.* 43, 254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-acetyltransferase 2

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(NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. At the other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of NgR protein, expression of NgR nucleic acid, or mutation content of NgR genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a NgR modulator, such as a modulator identified by one of the exemplary screening assays described herein.

Monitoring Clinical Efficacy

Monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of NgR (*e.g.*, the ability to modulate aberrant cell proliferation and/or differentiation) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase NgR gene expression, protein levels or upregulate NgR activity, can be monitored in clinical trials of subjects exhibiting decreased NgR gene

expression, protein levels, or downregulated NgR activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease NgR gene expression, protein levels, or downregulate NgR activity, can be monitored in clinical trials of subjects exhibiting increased NgR gene expression, protein levels, or upregulated NgR activity. In such clinical trials, the expression or activity of NgR and, preferably, other genes that have been implicated in, for example, a disease or disorder, can be used as a "read out" or markers of the immune responsiveness of a particular cell.

For example, genes, including NgR, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) that modulates NgR activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on demyelination disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of NgR and other genes implicated in the disorder. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced by one of the methods as described herein or by measuring the levels of activity of NgR or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the agent.

In one embodiment, the invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, protein, peptide, peptidomimetic, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a NgR protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the NgR protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the NgR protein, mRNA or genomic DNA in the pre-administration sample with the NgR protein, mRNA or genomic DNA in the post

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administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of NgR to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration
5 of the agent may be desirable to decrease expression or activity of NgR to lower levels than detected, *i.e.*, to decrease the effectiveness of the agent.

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods
10 of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant NgR expression or activity.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) levels or biological activity may be treated with Therapeutics that antagonize (*i.e.*, reduce or inhibit) activity. Therapeutics that
15 antagonize activity may be administered in a therapeutic or prophylactic manner. Therapeutics that may be utilized include, but are not limited to, (i) a NgR polypeptide, or analogs, derivatives, fragments or homologs thereof; (ii) antibodies to a NgR peptide; (iii) nucleic acids encoding a NgR peptide; (iv) administration of antisense nucleic acid and nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous
20 insertion within the coding sequences of coding sequences to a NgR peptide) are utilized to "knockout" endogenous function of a NgR peptide by homologous recombination (see, *e.g.*, Capecchi (1989) *Science* 244, 1288-1292); or (v) modulators (*i.e.*, inhibitors, agonists and antagonists, including additional peptide mimetic of the invention or antibodies specific to a peptide of the invention) that alter the interaction
25 between a NgR peptide and its binding partner.

Diseases and disorders that are characterized by decreased (relative to a subject not suffering from the disease or disorder) levels or biological activity may be treated with Therapeutics that increase (*i.e.*, are agonists to) activity. Therapeutics that upregulate activity may be administered in a therapeutic or prophylactic manner.
30 Therapeutics that may be utilized include, but are not limited to, a NgR peptide, or analogs, derivatives, fragments or homologs thereof; or an agonist that increases bioavailability.

Increased or decreased levels can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a NgR peptide). Methods that are well-known within the art
5 include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.).

In one aspect, the invention provides a method for preventing, in a subject, a
10 disease or condition associated with an aberrant NgR expression or activity, by administering to the subject an agent that modulates NgR expression or at least one NgR activity. Subjects at risk for a disease that is caused or contributed to by aberrant NgR expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic
15 agent can occur prior to the manifestation of symptoms characteristic of the NgR aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of NgR aberrancy, for example, a NgR agonist or NgR antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

20 Another aspect of the invention pertains to methods of modulating NgR expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of NgR protein activity associated with the cell. An agent that modulates NgR protein activity can be an agent as described herein, such as a nucleic acid or a
25 protein, a naturally-occurring cognate ligand of a NgR protein, a peptide, a NgR peptidomimetic, or other small molecule. In one embodiment, the agent stimulates one or more NgR protein activity. Examples of such stimulatory agents include active NgR protein and a nucleic acid molecule encoding NgR that has been introduced into the cell. In another embodiment, the agent inhibits one or more NgR protein activity.
30 Examples of such inhibitory agents include antisense NgR nucleic acid molecules and anti-NgR antibodies. These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the

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agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a NgR protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described
5 herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) NgR expression or activity. In another embodiment, the method involves administering a NgR protein or nucleic acid molecule as therapy to compensate for reduced or aberrant NgR expression or activity.

10 **Gene Therapy**

Mutations in the NgR gene that result in loss of normal function of the NgR gene product underlie NgR human disease states. The invention comprehends gene therapy to restore NgR activity to treat those disease states. Delivery of a functional NgR gene to appropriate cells is effected *ex vivo*, *in situ*, or *in vivo* by use of vectors,
15 and more particularly viral vectors (*e.g.*, adenovirus, adeno-associated virus, or a retrovirus), or *ex vivo* by use of physical DNA transfer methods (*e.g.*, liposomes or chemical treatments). See, for example, Anderson (1998) *Nature*, supplement to 392(6679):25-20. For additional reviews of gene therapy technology see Friedmann (1989) *Science* 244, 1275-1281; Verma (1990) *Sci. Am.* 68-84; and Miller (1992)
20 *Nature* 357, 455-460. Alternatively, it is contemplated that in other human disease states, preventing the expression of, or inhibiting the activity of, NgR will be useful in treating disease states. It is contemplated that antisense therapy or gene therapy could be applied to negatively regulate the expression of NgR.

The present invention provides for both prophylactic and therapeutic methods
25 of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant NgR expression or activity.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) levels or biological activity may be treated with Therapeutics that antagonize (*i.e.*, reduce or inhibit) activity. Therapeutics that
30 antagonize activity may be administered in a therapeutic or prophylactic manner. Therapeutics that may be utilized include, but are not limited to, (i) a NgR polypeptide, or analogs, derivatives, fragments or homologs thereof; (ii) antibodies to a NgR

peptide; (iii) nucleic acids encoding a NgR peptide; (iv) administration of antisense nucleic acid and nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous insertion within the coding sequences of coding sequences to a NgR peptide) are utilized to "knockout" endogenous function of a NgR peptide by homologous recombination (see, *e.g.*, Capecchi (1989), above); or (v) modulators (*i.e.*, inhibitors, agonists and antagonists, including additional peptide mimetic of the invention or antibodies specific to a peptide of the invention) that alter the interaction between a NgR peptide and its binding partner.

10 Diseases and disorders that are characterized by decreased (relative to a subject not suffering from the disease or disorder) levels or biological activity may be treated with Therapeutics that increase (*i.e.*, are agonists to) activity. Therapeutics that upregulate activity may be administered in a therapeutic or prophylactic manner. Therapeutics that may be utilized include, but are not limited to, a NgR peptide, or analogs, derivatives, fragments or homologs thereof, or an agonist that increases
15 bioavailability.

Increased or decreased levels can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a NgR peptide). Methods that are well-known within the art
20 include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.).

In one aspect, the invention provides a method for preventing, in a subject, a
25 disease or condition associated with an aberrant NgR expression or activity, by administering to the subject an agent that modulates NgR expression or at least one NgR activity. Subjects at risk for a disease that is caused or contributed to by aberrant NgR expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic
30 agent can occur prior to the manifestation of symptoms characteristic of the NgR aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of NgR aberrancy, for example, a NgR agonist or

NgR antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

Another aspect of the invention pertains to methods of modulating NgR expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of NgR protein activity associated with the cell. An agent that modulates NgR protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of a NgR protein, a peptide, a NgR peptidomimetic, or other small molecule. In one embodiment, the agent stimulates one or more NgR protein activity. Examples of such stimulatory agents include active NgR protein and a nucleic acid molecule encoding NgR that has been introduced into the cell. In another embodiment, the agent inhibits one or more NgR protein activity. Examples of such inhibitory agents include antisense NgR nucleic acid molecules and anti-NgR antibodies. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a NgR protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates or downregulates) NgR expression or activity. In another embodiment, the method involves administering a NgR protein or nucleic acid molecule as therapy to compensate for reduced or aberrant NgR expression or activity.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figure. Such modifications are intended to fall within the scope of the appended claims.

The following Table 5 contains the sequences of exemplary polynucleotides and polypeptides of the invention.

TABLE 5

The following DNA sequence NgR2 <SEQ ID NO. 1> was identified in humans:

5 ATGCTGCCCCGGGCTCAGGCGCCTGCTGCAAGCTCCCGCCTCGGCCTGCCTCCTGCTGATG
CTCCTGGCCCTGCCCCTGGCGGCCCCCAGCTGCCCCATGCTCTGCACCTGCTACTCATCC
CCGCCCACCGTGAGCTGCCAGGCCAACAACTTCTCCTCTGTGCCGCTGTCCCTGCCACCC
AGCACTCAGCGACTCTTCCTGCAGAACAACTCATCCGCACGCTGCGGCCAGGCACCTTT
GGGTCCAACCTGCTCACCTGTGGCTCTTCTCCAACAACCTCTCCACCATCTACCCGGGC
10 ACTTCCGCCACTTGCAAGCCCTGGAGGAGCTGGACCTCGGTGACAACCGGCACCTGCGC
TCGCTGGAGCCCCGACACCTTCCAGGGCCTGGAGCGGCTGCAGTCGCTGCATTGTACCGC
TGCCAGCTCAGCAGCCTGCCCGGCAACATCTTCCGAGGCCTGGTCAGCCTGCAGTACCTC
TACCTCCAGGAGAACAGCCTGCTCCACCTACAGGATGACTTGTTCGCGGACCTGGCCAAC
CTGAGCCACCTCTTCTCCACGGGAACCGCCTGCGGCTGCTCACAGAGCACGTGTTTCGC
15 GGCCTGGGCAGCCTGGACCGGCTGCTGCTGCACGGGAACCGGCTGCAGGGCGTGACCGC
GCGGCTTCCGCGGCCTCAGCCGCCTACCATCTCTACCTGTTCAACAACAGCCTGGCC
TCGCTGCCCCGGCGAGGCGCTCGCCGACCTGCCCTCGCTCGAGTTCCTGCGGCTCAACGCT
AACCCTGGGCGTGCGACTGCCGCGCGCGGCCGCTCTGGGCTGGTTCCAGCGCGCGCGC
GTGTCCAGCTCCGACGTGACCTGCGCCACCCCCCGGAGCGCCAGGGCCGAGACCTGCGC
20 GCGCTCCGCGAGGCCGACTTCCAGGCGTGTCCGCCCCGCGGCACCCACGCGGCCGGGCAGC
CGCGCCCCGCGGAACAGCTCCTCCAACACCTGTACGGGGTGGCCGAGGCCGGGGCGCCC
CCAGCCGATCCCTCCACCTCTACCGAGATCTGCCTGCCGAAGACTCGCGGGGGCGCCAG
GGCGGGGACGCGCTACTGAGGACGACTACTGGGGGGGTACGGGGGTGAGGACCAGCGA
GGGGAGCAGATGTGCCCCGGCGCTGCCTGCCAGGCGCCCCCGGACTCCCGAGGCCCTGCG
25 CTCTCGGCCGGGCTCCCCAGCCCTCTGCTTTGCCCTCCTGCTCCTGGTGCCCCACCACTC

The following amino acid sequence <SEQ ID NO. 2> is the predicted amino acid sequence derived from the DNA sequence of SEQ ID NO. 1:

30 M L P G L R R L L Q A P A S A C L L L M L L A L P L A A P S C
P M L C T C Y S S P P T V S C Q A N N F S S V P L S L P P S T
Q R L F L Q N N L I R T L R P G T F G S N L L T L W L F S N N
L S T I Y P G T F R H L Q A L E E L D L G D N R H L R S L E P
D T F Q G L E R L Q S L H L Y R C Q L S S L P G N I F R G L V
S L Q Y L Y L Q E N S L L H L Q D D L F A D L A N L S H L F L
35 H G N R L R L L T E H V F R G L G S L D R L L L H G N R L Q G
V H R A A F R G L S R L T I L Y L F N N S L A S L P G E A L A
D L P S L E F L R L N A N P W A C D C R A R P L W A W F Q R A
R V S S S D V T C A T P P E R Q G R D L R A L R E A D F Q A C
P P A A P T R P G S R A R G N S S S N H L Y G V A E A G A P P
40 A D P S T L Y R D L P A E D S R G R Q G G D A P T E D D Y W G
G Y G G E D Q R G E Q M C P G A A C Q A P P D S R G P A L S A
G L P S P L L C L L L L V P H H L

The following DNA sequence NgR3 <SEQ ID NO. 3> was identified in mouse:

5 ATGTCTTGGCAGTCTGGAACACAGTGACACAATCTCCCGTGCAGGCTGCTCAGGTCTCA
GGGTGCTGTGTGGAATTGCTGCTGTTGCTGCTCGCTGGAGAGCTACCTCTGGGTGGTGGT
TGTCTCGAGACTGTGTGTGCTACCCCTGCGCCCATGACTGTCAGCTGCCAGGCACACAAC
10 TTTGCTGCCATCCCGGAGGGCATCCCAGAGGACAGTGAGCGCATCTTCCTGCAGAACAAT
CGCATCACCTTCCTCCAGCAGGGCCACTTCAGCCCCGCCATGGTCACCCTCTGGATCTAC
TCCAACAACATCACTTTTATTGCTCCCCAACCTTCGAGGGCTTTGTGCATCTGGAGGAG
CTAGACCTTGGAGACAACCGACAGCTGCGAACGCTGGCACCCGAGACCTTCCAAGGCCTG
GTGAAGCTTCACGCCCTCTACCTCTATAAGTGTGGACTGAGCGCCCTGCCCCGAGGCATC
15 TTTGGTGGCCTGCACAGCCTGCAGTATCTCTACTTGCAGGACAACCATATCGAGTACCTC
CAAGATGACATCTTTGTGGACCTGGTCAATCTCAGTCACTTGTCTTCTCCATGGTAACAAG
CTATGGAGCCTGGGCCAAGGCATCTTCCGGGGCCTGGTGAACCTGGACCGGTTGCTGCTG
CATGAGAACCAGCTACAGTGGGTTCACCACAAGGCTTTCCATGACCTCCACAGGCTAACC
ACCTCTTTCTCTTCAACAACAGCCTCACTGAGCTGCAGGGTGACTGTCTGGCCCCCTG
20 GTGGCCTTGGAGTTCCCTTCGCCCTCAATGGGAATGCTTGGGACTGTGGCTGCCGGGCACGT
TCCCTGTGGGAATGGCTGCGAAGGTTCCGTGGCTCTAGCTCTGCTGTCCCCCTGCGCGACC
CCCCGAGCTGCGGCAAGGCCAGGATCTGAAGCTGCTGAGGGTGGAGGACTTCCGGAAGTGC
ACAGGACCAAGTGTCTCTCACCAGATCAAGTCTCACACGCTTACCACCTCTGACAGGGCT
GCCCCGAAGGAGCACCATCCGTCCCATGGGGCCTCCAGGGACAAAGGCCACCCACATGGC
25 CATCCGCCTGGCTCCAGGTCAAGTTACAAGAAGGCAGGCAAGAACTGCACCAGCCACAGG
AACCGGAACCAGATCTCTAAGGTGAGCTCTGGGAAAGAGCTTACCGAACTGCAGGACTAT
GCCCCGACTATCAGCACAAGTTTCACTTTGACATCATGCCACCCGACGACCCAAGAGG
AAGGGCAAGTGTGCTCGCAGGACCCCCATCCGTGCCCCAGTGGGGTGCAGCAGGCATCC
TCAGGCACGGCCCTTGGGGCCCCACTCCTGGCCTGGATACTGGGGCTGGCAGTCACTCTC
CGC

The following protein sequence <SEQ ID NO. 4> is deduced protein of SEQ ID NO:3:

30 MSWQSGTTVTQSPVQAAQVSGCCVELLLLLL
AGELPLGGGCPRDCVCYPAPMTVSCQAHNFA
AIPEGIPEDSERIFLQNNRITFLQQGHFSPA
MVTLWIYSNNITFIAPNTFEGFVHLEELDLG
DNRQLRTLAPETFQGLVKLHALYLYKCLSA
LPAGIFGGLHSLQYLYLQDNHIEYLQDDIFV
35 DLVNLSHLFLHGNKLWSLGQGIFRGLVNLDL
LLLHENQLQWVHHKAFHDLHRLTTLFLFNNS
LTELQGDCLAPLVALEFLRLNGNAWDCGCRA
RSLWEWLRRFRGSSSAVPCATPELRQGQDLK
LLRVEDFRNCTGPVSPHQIKSHTLTTSDDRAA
RKEHHPHSHGASRDKGHPHGHPPGSRSGYKKA
40 GKNCTSHRNRNQISKVSSGKELTELQDYAPD
YQHKFSFDIMPTARPKRKKGKCARRTPIRAPS
GVQQASSGTALGAPLLAWILGLAVTLR

The following protein sequence <SEQ ID NO. 5> is NgR1 from humans:

5 MKRASAGGSRL LA WVLWLQAWQVAAPCPGA
C
CYNEPKVTTSCPQQGLQAVPVGIPAA SQRI
FLHG NRISHVPAASFRA CRNLTLWLHS NVL
ARIDAA AFTGLALLEQLDLSDNAQLRSV DPA
TFHGLGRLHTLHLDR CGLQELGPGLFRGLAA
LQYLYLQDNALQALPDDTFRDLGNLTHLFLH
GNRISSVPERAFRGLHSLDRLLLHQNRVAHV
10 HPHA FRDLGRLMTLYLFANNLSALPTEALAP
LRA LQYLRRLNDNPWVCD CRARPLWAWLQKFR
GSSSEVP CSLPQRLAGRDLKRLAANDLQGCA
VATGPYHPIWTGRATDEEPLGLPKCCQPDAA
DKASVLEPGRPASAGNALKGRVPPGDSPPGN
15 GSGPRHINDSPFGTLPGSAEPPLTAVRPEG S
EPPGFPTSGPRRRPGCSRKNRTRSHCRLGQA
GSGGGGTGDSESGALPSLTCSLTPLGLALV
LWTVLGPC

The following amino acid sequence <SEQ ID NO:6> is a Consensus Sequence of
20 NgR based on homology with NgR1

25 C P X X C X C Y X X P X X T X S C X X X X X X X X P X
XXPXXXXRXFLXXNXIXXXXXXXXXXFXXXXXXXXXXLWX
XSNXXXXXIXXXXXFXXXXXXLEXLDLXDNXXLR
XXXPXTFXGLXXLXLXLXXCXLXXLXXXXFX
GLXXLQYLYLQXNX XXXLXDDXFXDLXNLXH
LFLHG NX XXXXXXXXXXXXFRGLXXLDRLLLHXNX
XXXVHXXAFXXLXRLXXLXLFXNXLXXLXXX
XLAXLXXLXXLR LNXXNXWXCXCRARXLWXWX
30 XXXRXSSSXVXCXXPXXXXXGXDLXXLXXXDX
XXCXXXXXPXPXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXGXXXXXXXXXXXXX
PPXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
35 XXXXXXXXXXXXXXXXXXXXXXXXXXXLXXXXX
XX X X X L

The following protein sequence <SEQ ID NO:7> is the 66 amino acid active domain
of Nogo:

40 R I Y K G V I Q A I Q K S D E G H P F R A Y L E S E V A I S E
E L V Q K Y S N S A L G H V N C T I K E L R R L F L V D D L V
D S L K

The following protein sequence <SEQ ID NO:8> is the amino acid sequence of the mature NgR2:

```

5  C P M L C T C Y S S P P T V S C Q A N N F S S V P L S L P P S
   T Q R L F L Q N N L I R T L R P G T F G S N L L T L W L F S N
   N L S T I Y P G T F R H L Q A L E E L D L G D N R H L R S L E
   P D T F Q G L E R L Q S L H L Y R C Q L S S L P G N I F R G L
   V S L Q Y L Y L Q E N S L L H L Q D D L F A D L A N L S H L F
   L H G N R L R L L T E H V F R G L G S L D R L L L H G N R L Q
   G V H R A A F R G L S R L T I L Y L F N N S L A S L P G E A L
10  A D L P S L E F L R L N A N P W A C D C R A R P L W A W F Q R
   A R V S S S D V T C A T P P E R Q Q R D L R A L R E A D F Q A
   C P P A A P T R P G S R A R G N S S S N H L Y G V A E A G A P
   P A D P S T L Y R D L P A E D S R G R Q G G D A P T E D D Y W
15  G G Y G G E D Q R G E Q M C P G A A C Q A P P D S R G P A L S
   A G L P S P L L C L L L L V P H H L

```

The following protein sequence <SEQ ID NO:9> is the amino acid sequence of the mature NgR3:

```

20  C P R D C V C Y P A P M T V S C Q A H N F A A I P E G I P E D
   S E R I F L Q N N R I T F L Q Q G H F S P A M V T L W I Y S N
   N I T F I A P N T F E G F V H L E E L D L G D N R Q L R T L A
   P E T F Q G L V K L H A L Y L Y K C G L S A L P A G I F G G L
   H S L Q Y L Y L Q D N H I E Y L Q D D I F V D L V N L S H L F
   L H G N K L W S L G Q G I F R G L V N L D R L L L H E N Q L Q
25  W V H H K A F H D L H R L T T L F L F N N S L T E L Q G D C L
   A P L V A L E F L R L N G N A W D C G C R A R S L W E W L R R
   F R G S S S A V P C A T P E L R Q G Q D L K L L R V E D F R N
   C T G P V S P H Q I K S H T L T T S D R A A R K E H H P S H G
   A S R D K G H P H G H P P G S R S G Y K K A G K N C T S H R N
30  R N Q I S K V S S G K E L T E L Q D Y A P D Y Q H K F S F D I
   M P T A R P K R K G K C A R R T P I R A P S G V Q Q A S S G T
   A L G A P L L A W I L G L A V T L R

```

The following amino acid sequence <SEQ ID NO:10> is a conserved cysteine motif (Cysteine domain 1) of the NgR and homologs based on the Consensus Sequence:

CPXXCXC YXXPXXTXSC

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The following amino acid sequence <SEQ ID NO:11> is a conserved cysteine motif (Cysteine domain 2) of the NgR and homologs based on the Consensus Sequence:

N X W X C X C R A R X L W X W X X X R X S S S X V X C X X P
X X X X G X D L X X L X X X D X X X C

The following amino acid sequence <SEQ ID NO:12> is a conserved Leucine-rich domain of the NgR and homologs based on the Consensus Sequence:

R X F L X X N X I X X X X X X F X X X X X X X L W X X S N
X X X X I X X X X F X X X X X L E X L D L X D N X X L R X X X
P X T F X G L X X L X L X L X X C X L X X L X X X F X G L X
X L Q Y L Y L Q X N X X X X L X D D X F X D L X N L X H L F L
H G N X X X X X X X X F R G L X X L D R L L L H X N X X X X
V H X X A F X X L X R L X X L X L F X N X L X X L X X X X L A
X L X X L X X L R L

Unless otherwise indicated, X is any amino acid. For example, X where indicated may be no amino acid. Additional features of the invention will be apparent from the following Examples. Examples 1-5 are actual, while the remaining Examples are prophetic.

As shown by the following Examples, a gene encoding novel NgRs have been identified by computational analysis of DNA sequence data. The proteins encoded by NgR2 and NgR3 have a putative signal sequence, eight leucine-rich repeat domains in a conserved leucine-rich region (SEQ ID NO:12), a conserved cysteine-rich region (SEQ ID NO:10) N-terminal to the leucine-rich region, a second cysteine-rich domain (SEQ ID NO:11) C-terminal to the leucine-rich region, and a putative glycosylphosphatidylinositol-linkage (GPI-linkage) site. NgR2 and NgR3 differ from the previously identified NgR sequence. The NgR homologs, when compared to known NgRs, show a consensus sequence (SEQ ID NOs:6). The putative mature NgR2 and NgR3 are shown in Table 5 as SEQ ID NOs: 8 and 9, respectively.

Example 1: Tblastn query of the HTG database

The protein sequence for the human NgR (NgR1) (SEQ ID NO:5) was used to query the high throughput genomic (HTG) database the use of which is familiar to those skilled in the art. The HTG database is a part of GenBank, a comprehensive

NIH genetic sequence database, which includes an annotated collection of all publicly available DNA sequences (*Nucleic Acids Res.* (2000) 28, 15-8). The HTG database includes sequences obtained from genomic DNA. Within genomic DNA, genes are typically encoded by multiple segments of DNA called exons. Thus when one aligns a cDNA sequence (or a protein sequence encoded by a cDNA sequence) to a genomic sequence, the sequence will be broken up into segments depending on the number of exons in the gene.

The BLAST algorithm, which stands for Basic Local Alignment Search Tool is suitable for determining sequence similarity (Altschul *et al.*, (1990) *J. Mol. Biol.* 215, 403-410, which is incorporated herein by reference in its entirety). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The basic BLAST algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension for the word hits in each direction are halted when: 1) the cumulative alignment score falls off by the quantity X from its maximum achieved value; 2) the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or 3) the end of either sequence is reached. The Blast algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The Blast program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89, 10915-10919, which is incorporated herein by reference in its entirety) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm (Karlin *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 5873-5877, which is incorporated herein by reference) and Gapped BLAST perform a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which

provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a NgR gene or cDNA if the smallest sum probability in comparison of the test nucleic acid to a NgR nucleic acid is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

To query the HTG database with the NgR protein sequence, we used a variation of the BLAST algorithm known as the tblastn program, which compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames (*J. Mol. Biol.* (1990) 215, 403-410; *Nucleic Acids Res.* (1997) 25, 3389-3402). The results of the tblastn search indicated the presence of genes in the database with a significant identity to the NgR. In addition to finding hits to genomic clones which contain the human and mouse NgR genes, we found hits to clones where the identity was not as high, but still very significant. Three human clones were found (Accession numbers: AC068514, AC016869, AC013606) with an e-value of 4e-43 and one mouse clone was found (Accession No. AC021768) with an e-value of 1e-78. The three human clones all appeared to encode the same gene, so further analysis was confined to AC013606.

20 **Example 2: Prediction of the human NgR2 protein sequence (AC013606)**

The human NgR protein sequence aligned with two regions of translated sequence from nucleotide sequence AC013606, indicating that the new gene was encoded by at least two exons. In order to define the complete gene, we used the computer program GENSCAN[™] (*J. Mol. Biol.* (1997) 268, 78-94) which can identify complete exon/intron structures of genes in genomic DNA. The gene prediction by GENSCAN[™] contained seven exons. By comparing these predicted exons to the NgR, it was concluded that the new human gene contains two of these exons and a part of another (containing the initiating methionine). The predicted cDNA (mRNA) encoded by these three exons was assembled from AC013606 (HTG11; deposited March 2000; length = 143899; GenBank release 118.0; SEQ ID NO: 15) by combining nucleotides from the three exons whose coordinates are: 123292-123322 (exon 1); 130035-130516 (exon 2); and 138589-139335 (exon 3). The sequence for this cDNA

sequence is SEQ ID NO:1 (nucleotide sequence of human NgR2; AC013606). The translation of this cDNA provides the protein sequence of human NgR2 (SEQ ID NO:2).

We used the protein sequence of human NgR2 as a query sequence against the human EST database. A number of hits of high significance were found indicating that the NgR2 mRNA is expressed in a number of tissues including fetal brain. Furthermore, two of these ESTs provided support for the exon structure that we deduced. One EST (Accession No: GB_EST19:AI346757) contains 565 nucleotides corresponding to amino acids 84-271 of the human NgR2 (SEQ ID No:4). This spans the second intron located between amino acids 171 and 172, and provides positive evidence for the splicing of exons 2 and 3 at the mRNA level. Another EST (GB_EST26:AI929019) contains 545 nucleotides, part of which corresponds to amino acids 1-75 of the human NgR2 (SEQ ID NO:2). This spans the first intron located between amino acids 10 and 11, and provides positive evidence for the splicing of exons 1 and 2 at the mRNA level.

Example 3: Prediction of the mouse NgR3 protein sequence (AC021768)

The human NgR protein sequence aligned with only one region of translated sequence from nucleotide sequence AC021768, indicating that most of the new mouse gene was encoded by one large exon. However, upon inspection, the protein encoded by this exon was missing an initiating methionine. In order to define the complete gene, we used the computer program GENSCAN as described above. The gene prediction by GENSCAN contained two exons; the large one found by visual inspection and a short one at the 5' end which provided an initiating methionine. The predicted cDNA (mRNA) encoded by these two exons was assembled from AC021768 (HTG14; deposited March 2000; length = 215980; GenBank release 118.0; SEQ ID NO: 16) by combining nucleotides from the two exons whose coordinates are: the complement of 164265-164325 (exon 1); and the complement of 155671-156992 (exon 2). The sequence for this cDNA sequence is SEQ ID NO:3 (nucleotide sequence of mouse NgR3; AC021768). The translation of this cDNA provides the protein sequence of mouse NgR3 (SEQ ID NO:4).

We used the protein sequence of mouse NgR3 as a query sequence against the mouse EST database. One hit of high significance was found indicating that the NgR2 mRNA is expressed in the heart. This EST (GB_EST20:AI428334) contains 463 nucleotides, part of which correspond to amino acids 45-193 of mouse NgR3 (SEQ ID NO:4).

Example 4: Similarity between the NgRs

An alignment between NgR1 and the two new receptors is shown in Fig. 1A-1B. The similarities between these proteins include:

- (1) The SignalP program, which locates the signal sequence cleavage position, predicts a cleavage before the first conserved cysteine in all the proteins. Thus the mature protein in all cases will have a cysteine at the N-terminus.
- (2) All proteins contain eight Leucine Rich Repeats (LRR). LRRs are short sequence motifs present in a number of proteins with diverse functions and cellular locations. These repeats are usually involved in protein-protein interactions. Each LRR is composed of a beta-alpha unit.
- (3) All three proteins contain a leucine rich repeat N-terminal domain (LRRNT), in which four cysteines are conserved. LRRs are often flanked by cysteine rich domains at both their N and C termini.
- (4) All three proteins contain a LRR C-terminal domain (LRRCT). The LRRCTs of the three NgR proteins can be distinguished from those of other LRR containing proteins, by the pattern of typtophans and cysteines which are completely conserved in this domain.
- (5) All three proteins contain a conserved cysteine in the fourth LRR domain.
- (6) All three proteins contain a conserved potential glycosylation site in the eighth LRR domain.
- (7) NgR2 and NgR3 have a hydrophobic C-terminus, as does NgR1, an indication that they probably also undergo a modification similar to NgR1, where a GPI moiety is covalently linked to a C-terminal amino acid. This allows the protein to remain tethered to the cell.

Example 5: Preparation of Nogo Proteins

A Nogo binding assay was developed which utilizes a method widely used in examining semaphorin and ephrin axonal guidance function (Flanagan & Vanderhaeghen (1998) *Annu. Rev. Neurosci.* 21,3 09-345; Takahashi *et al.*, (1999) *Cell* 99, 59-69). It involves fusing a secreted placental alkaline phosphatase (AP) moiety to the ligand in question to provide a biologically active receptor binding agent which can be detected with an extremely sensitive colorimetric assay. For Nogo, an expression vector is created encoding a signal peptide, a His6 tag for purification, AP, and the 66 amino acid active domain of Nogo. The fusion protein can be purified from the conditioned medium of transfected cells in milligram amounts. This protein is biologically active as a growth cone collapsing agent with an EC₅₀ of 1 nM.

Alternatively, a glutathione-S-transferase Nogo (GST-Nogo) fusion protein may be prepared. For GST-Nogo, an expression vector (*e.g.*, a pGEX vector) is created encoding a signal peptide, GST, and the 66 amino acid active domain of Nogo. GST-Nogo may be purified from the culture medium and used as a GST fusion protein, or GST may be cleaved from the Nogo portion of the fusion protein with an enzyme that recognizes the specific amino acid cleavage site engineered between the GST portion and the Nogo portion of the fusion protein. Such sites are part of the commercially available GST vectors. The specific cleavage sites and enzymes may be used in accordance with the Manufacturer's specifications.

It has been found that AP-Nogo is actually slightly more potent than GST-Nogo, perhaps because the protein is synthesized in a eukaryotic rather than a prokaryotic cell.

Binding of Nogo to immobilized NgR homologs may be performed in an ELISA-type assay in which AP-Nogo is allowed to react with an immobilized receptor homolog. Specificity of binding may be demonstrated in a competitive binding assay using increasing amounts of GST-Nogo in the type of assay to show a decreasing amount of binding of AP-Nogo (as judged in the colorimetric assay).

Example 6: Transfected COS Cell binding Assays

The homologs of the present invention may be used in transfection studies in COS cells to demonstrate binding of Nogo. Specifically, nucleotide sequences

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encoding NgR2 and NgR3 may be transfected into COS cells using a suitable vector. Non-transfected COS-7 cells do not bind AP-Nogo. However, transfection of COS cells with nucleic acid sequences encoding NgRs will make them capable of binding Nogo. AP alone does not bind with any stable affinity to these transfected cells, indicating that any affinity of Nogo for NgR2 or NgR3 would be due to the 66 amino acids derived from Nogo. Furthermore, specific affinity of Nogo for the NgR2 or NgR3 proteins may be tested in displacement of AP-Nogo assays using GST-Nogo. NgR2 and/or NgR3 may also bind homologs of Nogo, which may also be tested using this assay.

Example 7: Expression of NgR in Human Cell Lines using Northern Blot and a Random-Primed Probe

A Northern blot is purchased from a commercial source, or RNA samples from cells of interest are run on an agarose gel and blotted to a membrane using any of the well known techniques for Northern blotting. The blot is probed with a fragment of NgR2 (SEQ ID NO:1) or NgR3 (SEQ ID NO:3). The probe is prepared from 50 ng of cDNA labeled by a random-primed method (Feinberg and Vogelstein (1983) *Anal. Biochem.* 132, 6-13). Hybridization is carried out at 68°C for 1 hour in ExpressHyb™ solution (Clontech, Cat. No. 8015-1) followed by washing with 2X SSC/0.05% SDS at room temperature and two washes with 0.1X SSC/0.1% SDS at 50°C. Expression of NgR2 and/or NgR3 can be assessed by the presence of an appropriately sized band on the blot.

Example 8: Cloning of cDNA corresponding to NgRs

To obtain the full-length clone corresponding to NgR2 from a cDNA library, the following method may be used. A cDNA library is generated using standard methods from a tissue known to contain NgR2. Such a tissue was identified in Example 2. 1×10^6 plaque forming units from the cDNA library may be screened in duplicate on OPTITRAN™ filters. The filters are hybridized with ³²P-labeled oligonucleotides that are generated from the ESTs corresponding to portions of NgR2. The hybridization reaction may consist of 400 mls plaque screen buffer (50mM Tris pH

7.5, 1M NaCl, 0.1% Sodium pyrophosphate, 0.2% Polyvinylpyrrolidone and 0.2% Ficoll) containing 10% Dextran sulfate and 100µg/ml tRNA and 80 pmol each ³²P-labeled oligonucleotide at 65°C overnight. The filters are washed twice with 2X SSC/1%SDS and twice with 1X SSC/1%SDS and exposed to film. Duplicate positives are purified. DNA from each of these clones is analyzed by restriction enzyme digest followed by agarose gel electrophoresis and Southern blotting. The filters are hybridized to the ³²P-labeled oligonucleotides used for the original hybridization to confirm that inserts hybridize to the probe. The insert is then sequenced to confirm that it represents the cDNA for NgR2. Similar methods may be used to generate a full-length clone corresponding to NgR3.

Alternatively, a full-length clone of NgR2 or NgR3 can be obtained by a person of ordinary skill in the art employing conventional PCR techniques.

Example 9: Hybridization Analysis to demonstrate NgR expression in the brain

The expression of NgR in mammals, such as the rat, may be investigated by *in situ* hybridization histochemistry. To investigate expression in the brain, for example, coronal and sagittal rat brain cryosections (20 µm thick) are prepared using a Reichert-Jung cryostat. Individual sections are thaw-mounted onto silanized, nuclease-free slides (CEL Associates, Inc., Houston, TX), and stored at -80°C. Sections are processed starting with post-fixation in cold 4% paraformaldehyde, rinsed in cold phosphate-buffered saline (PBS), acetylated using acetic anhydride in triethanolamine buffer, and dehydrated through a series of alcohol washes in 70%, 95%, and 100% alcohol at room temperature. Subsequently, sections are delipidated in chloroform, followed by rehydration through successive exposure to 100% and 95% alcohol at room temperature. Microscope slides containing processed cryosections are allowed to air dry prior to hybridization. Other tissues may be assayed in a similar fashion.

A NgR-specific probe may be generated using PCR. Following PCR amplification, the fragment is digested with restriction enzymes and cloned into pBluescript II cleaved with the same enzymes. For production of a probe specific for the sense strand of NgR, a cloned NgR fragment cloned in pBluescript II may be linearized with a suitable restriction enzyme, which provides a substrate for labeled run-off transcripts (*i.e.*, cRNA riboprobes) using the vector-borne T7 promoter and

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commercially available T7 RNA polymerase. A probe specific for the antisense strand of NgR may also be readily prepared using the NgR clone in pBluescript II by cleaving the recombinant plasmid with a suitable restriction enzyme to generate a linearized substrate for the production of labeled run-off cRNA transcripts using the T3 promoter and cognate polymerase. The riboprobes may be labeled with [³⁵S]-UTP to yield a specific activity of about 0.40 x 10⁶ cpm/pmol for antisense riboprobes and about 0.65 x 10⁶ cpm/pmol for sense-strand riboprobes. Each riboprobe may be subsequently denatured and added (2 pmol/ml) to hybridization buffer which contains 50% formamide, 10% dextran, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 1X Denhardt's Solution, and 10 mM dithiothreitol. Microscope slides containing sequential brain cryosections may be independently exposed to 45 µl of hybridization solution per slide and silanized cover slips may be placed over the sections being exposed to hybridization solution. Sections are incubated overnight (15-18 hours) at 52°C to allow hybridization to occur. Equivalent series of cryosections are then exposed to sense or antisense NgR-specific cRNA riboprobes.

Following the hybridization period, coverslips are washed off the slides in 1X SSC, followed by RNase A treatment involving the exposure of slides to 20 µg/ml RNase A in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.5 M EDTA, and 0.5 M NaCl for 45 minutes at 37°C. The cryosections are then subjected to three high-stringency washes in 0.1 X SSC at 52°C for 20 minutes each. Following the series of washes, cryosections are dehydrated by consecutive exposure to 70%, 95%, and 100% ammonium acetate in alcohol, followed by air drying and exposure to Kodak BioMax™ MR-1 film. After 13 days of exposure, the film is developed, and any significant hybridization signal is detected. Based on these results, slides containing tissue that hybridized, as shown by film autoradiograms, are coated with Kodak NTB-2 nuclear track emulsion and the slides are stored in the dark for 32 days. The slides are then developed and counterstained with hematoxylin. Emulsion-coated sections are analyzed microscopically to determine the specificity of labeling. The signal is determined to be specific if autoradiographic grains (generated by antisense probe hybridization) are clearly associated with cresyl violet-stained cell bodies. Autoradiographic grains found between cell bodies indicate non-specific binding of the probe.

In some cases, such as using a probe to detect a NgR homolog in a heterologous species, in order to achieve optimal hybridization, it may be necessary to decrease the stringency conditions. Such conditions are well known to those of ordinary skill in the art and examples are provided above.

5 Expression of NgR in the brain provides an indication that modulators of NgR activity have utility for treating neurological disorders. Some other diseases for which modulators of NgR may have utility include depression, anxiety, bipolar disease, epilepsy, neuritis, neurasthenia, neuropathy, neuroses, and the like. Use of NgR modulators, including NgR ligands and anti-NgR antibodies, to treat individuals having
10 such disease states is intended as an aspect of the invention.

Example 10: Northern Blot Analysis of NgR-RNA with a PCR-generated Probe

Northern blot hybridizations may be performed to examine the expression of NgR mRNA. A clone containing at least a portion of the sequence of SEQ ID NO:1
15 may be used as a probe. Vector-specific primers are used in PCR to generate a hybridization probe fragment for ³²P-labeling. The PCR is performed as follows:

20	Mix:	1μl	NgR-containing plasmid
		2μl	fwd primer (10-50 pM)
		2μl	rev primer (10-50 pM)
		10μl	10xPCR buffer (such as that provided with the enzyme, Amersham Pharmacia Biotech)
		1μl	10mM dNTP (such as #1 969 064 from Boehringer Mannheim)
25		0.5μl	Taq polymerase (such as #27-0799-62, Amersham Pharmacia Biotech)
		83.5μl	water

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PCR is performed in a Thermocycler using the following program:

	94°C	5min		
5	94°C	1min		30 cycles
	55°C	1min		
	72°C	1min		
10	72°C	10min		

The PCR product may be purified using QIAquick PCR Purification Kit (#28104) from Qiagen, and radiactively labeled with ³²P-dCTP (#AA0005/250, Amersham Pharmacia Biotech)) may be done by random priming using "Ready-to-go DNA Labeling Beads" (#27-9240-01) from Amersham Pharmacia Biotech.

Hybridization is carried out on Human Multiple Tissue Northern Blot from Clontech as described in manufacturer's protocol, or on a Northern Blot prepared by running RNA samples from cells of interest on an agarose gel and blotting to a membrane using any of the known Northern blotting protocols. After exposure overnight on Molecular Dynamics Phosphor Imager screen (#MD146-814) bands of an appropriate size are visualized.

Example 11: Recombinant Expression of NgR in Eukaryotic Host Cells

A. Expression of NgR in Mammalian Cells

To produce NgR protein, a NgR-encoding polynucleotide is expressed in a suitable host cell using a suitable expression vector and standard genetic engineering techniques. For example, a NgR-encoding sequence described in Table 4 is subcloned into the commercial expression vector pzeoSV2 (Invitrogen, San Diego, CA) and transfected into Chinese Hamster Ovary (CHO) cells using the transfection reagent FuGENE6™ (Boehringer-Mannheim) and the transfection protocol provided in the product insert. Other eukaryotic cell lines, including human embryonic kidney (HEK 293) and COS cells, are suitable as well. Cells stably expressing NgR are selected by growth in the presence of 100 µg/ml zeocin (Stratagene, LaJolla, CA). As an alternative to FuGENE6™, the expression vector may carry the gene for dihydrofolate reductase (dhfr) and selection of clones with methotrexate (MTX) drug pressure

allows for stable transformation of CHO cells. Optionally, NgR may be purified from the cells using standard chromatographic techniques. To facilitate purification, antisera is raised against one or more synthetic peptide sequences that correspond to portions of the NgR amino acid sequence, and the antisera is used to affinity purify Nogo-R.

- 5 The NgR also may be expressed in-frame with a tag sequence (*e.g.*, polyhistidine, hemagglutinin, FLAG) to facilitate purification. Moreover, it will be appreciated that many of the uses for NgR polypeptides, such as assays described below, do not require purification of NgR from the host cell.

10 **B. Expression of NgR in CHO cells**

- For expression of NgR in Chinese hamster ovary (CHO) cells, a plasmid bearing the relevant NgR coding sequence is prepared, using a vector which also bears the selectable marker dihydrofolate reductase (DHFR). The plasmid is transfected into CHO cells. Selection under MTX drug pressure allows for preparation of stable transformants of a NgR (NgR2 or NgR3) in an expression plasmid carrying a selectable marker such as DHFR.
- 15

C. Expression of NgR in 293 cells

- For expression of NgR in mammalian cells 293 (transformed human, primary embryonic kidney cells), a plasmid bearing the relevant NgR coding sequence is prepared, using vector pSecTag2A (Invitrogen). Vector pSecTag2A contains the murine IgK chain leader sequence for secretion, the c-myc epitope for detection of the recombinant protein with the anti-myc antibody, a C-terminal polyhistidine for purification with nickel chelate chromatography, and a Zeocin resistant gene for selection of stable transfectants. The forward primer for amplification of this NgR cDNA is determined by routine procedures and preferably contains a 5' extension of nucleotides to introduce the HindIII cloning site and nucleotides matching the NgR sequence. The reverse primer is also determined by routine procedures and preferably contains a 5' extension of nucleotides to introduce an *XhoI* restriction site for cloning and nucleotides corresponding to the reverse complement of the NgR sequence. The PCR conditions are 55°C as the annealing temperature. The PCR product is gel purified and cloned into the *HindIII-XhoI* sites of the vector.
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- 25
- 30

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The DNA is purified using Qiagen chromatography columns and transfected into 293 cells using DOTAP™ transfection media (Boehringer Mannheim, Indianapolis, IN). Transiently transfected cells are tested for expression after 24 hours of transfection, using western blots probed with anti-His and anti-NgR peptide antibodies. Permanently transfected cells are selected with Zeocin and propagated. Production of the recombinant protein is detected from both cells and media by Western blots probed with anti-His, anti-Myc or anti-NgR peptide antibodies.

D. Transient Expression of Nogo-R in COS cells

For expression of the NgR in COS7 cells, a polynucleotide molecule having a nucleotide sequence of SEQ ID NO:1, for example, can be cloned into vector p3-CI. This vector is a pUC18-derived plasmid that contains the HCMV (human cytomegalovirus) promoter-intron located upstream from the bGH (bovine growth hormone) polyadenylation sequence and a multiple cloning site.

The forward primer is determined by routine procedures and preferably contains a 5' extension which introduces an *Xba*I restriction site for cloning, followed by nucleotides which correspond to a nucleotide sequence of SEQ ID NO:1. The reverse primer is also determined by routine procedures and preferably contains 5'-extension of nucleotides which introduces a *Sa*I cloning site followed by nucleotides which correspond to the reverse complement of a nucleotide sequence of SEQ ID NO:1.

The PCR consists of an initial denaturation step of 5 min at 95°C, 30 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 58°C and 30 sec extension at 72°C, followed by 5 min extension at 72°C. The PCR product is gel purified and ligated into the *Xba*I and *Sa*I sites of vector p3-CI. This construct is transformed into *E. coli* cells for amplification and DNA purification. The DNA is purified with Qiagen chromatography columns and transfected into COS 7 cells using Lipofectamine™ reagent from BRL, following the manufacturer's protocols. Forty-eight and 72 hours after transfection, the media and the cells are tested for recombinant protein expression.

NgR expressed from a COS cell culture can be purified by concentrating the cell-growth media to about 10 mg of protein/ml, and purifying the protein by, for

example, chromatography. Purified NgR is concentrated to 0.5 mg/ml in an Amicon concentrator fitted with a YM-10 membrane and stored at -80°C. NgR3 may also be expressed using this method and the nucleotide sequence of SEQ ID NO:3 or SEQ ID NO:13.

5

E. Expression of NgR in Insect Cells

For expression of NgR in a baculovirus system, a polynucleotide molecule having a nucleotide sequence of SEQ ID NO:1, 3 or 13 can be amplified by PCR. The forward primer is determined by routine procedures and preferably contains a 5' extension which adds the *NdeI* cloning site, followed by nucleotides which correspond to a nucleotide sequence of SEQ ID NO:1 (or SEQ ID NO:3 or SEQ ID NO:13, respectively). The reverse primer is also determined by routine procedures and preferably contains a 5' extension which introduces the *KpnI* cloning site, followed by nucleotides which correspond to the reverse complement of a nucleotide sequence of SEQ ID NO:1 (or SEQ ID NO:3 or SEQ ID NO:13, respectively).

The PCR product is gel purified, digested with *NdeI* and *KpnI*, and cloned into the corresponding sites of vector pACHTL-A (Pharmlngen, San Diego, CA). The pACHTL expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV), and a 6XHis tag upstream from the multiple cloning site. A protein kinase site for phosphorylation and a thrombin site for excision of the recombinant protein precede the multiple cloning site is also present. Of course, many other baculovirus vectors could be used in place of pACHTL-A, such as pAc373, pVL941 and pAcIM1. Other suitable vectors for the expression of NgR polypeptides can be used, provided that the vector construct includes appropriately located signals for transcription, translation, and trafficking, such as an in-frame AUG and a signal peptide, as required. Such vectors are described in Luckow *et al.*, Virology 170:31-39, among others.

The virus is grown and isolated using standard baculovirus expression methods, such as those described in Summers *et al.* (1987) A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experimental Station Bulletin No. 1555.

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In a preferred embodiment, pAcHLT-A containing NgR gene is introduced into baculovirus using the "BaculoGold™" transfection kit (Pharmingen, San Diego, CA) using methods established by the manufacturer. Individual virus isolates are analyzed for protein production by radiolabeling infected cells with ³⁵S-methionine at 24 hours post infection. Infected cells are harvested at 48 hours post infection, and the labeled proteins are visualized by SDS-PAGE. Viruses exhibiting high expression levels can be isolated and used for scaled up expression.

For expression of a NgR polypeptide in a Sf9 cells, a polynucleotide molecule having the nucleotide sequence of SEQ ID NO:1 (or SEQ ID NO:3 or SEQ ID NO:13) can be amplified by PCR using the primers and methods described above for baculovirus expression. The NgR cDNA is cloned into vector pAcHLT-A (Pharmingen) for expression in Sf9 insect. The insert is cloned into the *NdeI* and *KpnI* sites, after elimination of an internal *NdeI* site (using the same primers described above for expression in baculovirus). DNA is purified with Qiagen chromatography columns and expressed in Sf9 cells. Preliminary Western blot experiments from non-purified plaques are tested for the presence of the recombinant protein of the expected size which reacted with the NgR-specific antibody. These results are confirmed after further purification and expression optimization in HiG5 cells.

F. Expression of soluble forms of NgR2 and NgR3 as NgR-Ig fusion proteins.

To generate a NgR2-Ig fusion protein, standard methods may be used as described in the literature (e.g. Sanicola *et al.* (1997) *Proc. Natl. Acad. Sci. USA.* 94, 6238-6243). For example, a DNA fragment encoding NgR2 without the sequence encoding the hydrophobic C-terminus (GPI anchor signal) may be ligated to a DNA fragment encoding the Fc domain of IgG1 (which may be human IgG1), and the chimeric fragment may be cloned into an expression vector to generate a plasmid. The plasmid may then be transfected into Chinese hamster ovary cells to generate a stable cell line producing the fusion protein. The fusion protein is then purified from conditioned media using standard methods. For example, clarified conditioned media from the cell line may be loaded by gravity directly onto Protein A Sepharose. The column may then be washed with five column volumes each of PBS, PBS containing

0.5 M NaCl, and 25 mM sodium phosphate, 100 mM NaCl (pH 5.0). The bound protein may then be eluted with 25 mM NaH_2PO_4 , 100 mM NaCl (pH 2.8) and immediately neutralized with 1/10 fraction volume of 0.5 M Na_2HPO_4 (pH 8.6).

Similar methods may be used to generate a NgR3-Ig fusion protein.

5

Example 12: Interaction Trap/Two-Hybrid System

In order to assay for NgR-interacting proteins, the interaction trap/two-hybrid library screening method can be used. This assay was first described in Fields *et al.* (1989) *Nature* 340, 245, which is incorporated herein by reference in its entirety. A protocol is published in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY 1999, John Wiley & Sons, NY and Ausubel, F. M. *et al.* 1992, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, fourth edition, Greene and Wiley-interscience, NY, which is incorporated herein by reference in its entirety. Kits are available from Clontech, Palo Alto, CA (Matchmaker Two-Hybrid System 3).

15 A fusion of the nucleotide sequences encoding all or partial NgR and the yeast transcription factor GAL4 DNA-binding domain (DNA-BD) is constructed in an appropriate plasmid (*i.e.*, pGBKT7) using standard subcloning techniques. Similarly, a GAL4 active domain (AD) fusion library is constructed in a second plasmid (*i.e.*, pGADT7) from cDNA of potential NgR-binding proteins (for protocols on forming cDNA libraries, see Sambrook *et al.* 1989, MOLECULAR CLONING: A LABORATORY
20 MANUAL, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), which is incorporated herein by reference in its entirety. The DNA-BD/NgR fusion construct is verified by sequencing, and tested for autonomous reporter gene activation and cell toxicity, both of which would prevent a successful two-hybrid
25 analysis. Similar controls are performed with the AD/library fusion construct to ensure expression in host cells and lack of transcriptional activity. Yeast cells are transformed (ca. 10⁵ transformants/mg DNA) with both the NgR and library fusion plasmids according to standard procedure (Ausubel, *et al.*, 1992, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, fourth edition, Greene and Wiley-interscience, NY, which is
30 incorporated herein by reference in its entirety). *In vivo* binding of DNA-BD/NgR with AD/library proteins results in transcription of specific yeast plasmid reporter genes (*i.e.*, *lacZ*, *HIS3*, *ADE2*, *LEU2*). Yeast cells are plated on nutrient-deficient

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media to screen for expression of reporter genes. Colonies are dually assayed for β -galactosidase activity upon growth in Xgal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) supplemented media (filter assay for β -galactosidase activity is described in Breeden *et al.*, (1985) *Cold Spring Harb. Symp. Quant. Biol.*, 50, 643, which is incorporated herein by reference in its entirety).

5 Positive AD-library plasmids are rescued from transformants and reintroduced into the original yeast strain as well as other strains containing unrelated DNA-BD fusion proteins to confirm specific NgR/library protein interactions. Insert DNA is sequenced to verify the presence of an open reading frame fused to GAL4 AD and to determine

10 the identity of the NgR-binding protein.

Example 13: Antibodies to Nogo-R

Standard techniques are employed to generate polyclonal or monoclonal antibodies to the NgR receptor, and to generate useful antigen-binding fragments thereof or variants thereof, including "humanized" variants. Such protocols can be

15 found, for example, in Sambrook *et al.* (1989), above, and Harlow *et al.* (Eds.), ANTIBODIES A LABORATORY MANUAL; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988). In one embodiment, recombinant NgR polypeptides (or cells or cell membranes containing such polypeptides) are used as antigen to generate

20 the antibodies. In another embodiment, one or more peptides having amino acid sequences corresponding to an immunogenic portion of NgR (*e.g.*, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more amino acids) are used as antigen. Peptides corresponding to extracellular portions of Nogo-R, especially hydrophilic extracellular portions, are preferred. The antigen may be mixed with an adjuvant or linked to a

25 hapten to increase antibody production.

A. Polyclonal or Monoclonal antibodies

As one exemplary protocol, recombinant NgR or a synthetic fragment thereof is used to immunize a mouse for generation of monoclonal antibodies (or larger

30 mammal, such as a rabbit, for polyclonal antibodies). To increase antigenicity, peptides are conjugated to Keyhole Limpet Hemocyanin (Pierce), according to the manufacturer's recommendations. For an initial injection, the antigen is emulsified with

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Freund's Complete Adjuvant and injected subcutaneously. At intervals of two to three weeks, additional aliquots of NgR antigen are emulsified with Freund's Incomplete Adjuvant and injected subcutaneously. Prior to the final booster injection, a serum sample is taken from the immunized mice and assayed by western blot to confirm the presence of antibodies that immunoreact with NgR. Serum from the immunized animals may be used as polyclonal antisera or used to isolate polyclonal antibodies that recognize NgR. Alternatively, the mice are sacrificed and their spleen removed for generation of monoclonal antibodies.

To generate monoclonal antibodies, the spleens are placed in 10 ml serum-free RPMI 1640, and single cell suspensions are formed by grinding the spleens in serum-free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin (RPMI) (Gibco, Canada). The cell suspensions are filtered and washed by centrifugation and resuspended in serum-free RPMI. Thymocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a Feeder Layer. NS-1 myeloma cells, kept in log phase in RPMI with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT) for three days prior to fusion, are centrifuged and washed as well.

To produce hybridoma fusions, spleen cells from the immunized mice are combined with NS-1 cells and centrifuged, and the supernatant is aspirated. The cell pellet is dislodged by tapping the tube, and 2 ml of 37°C PEG 1500 (50% in 75 mM HEPES, pH 8.0) (Boehringer-Mannheim) is stirred into the pellet, followed by the addition of serum-free RPMI. Thereafter, the cells are centrifuged, resuspended in RPMI containing 15% FBS, 100 µM sodium hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer-Mannheim) and 1.5×10^6 thymocytes/ml, and plated into 10 Corning flat-bottom 96-well tissue culture plates (Corning, Corning, NY).

On days 2, 4, and 6 after the fusion, 100 µl of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusions are screened by ELISA, testing for the presence of mouse IgG that binds to NgR. Selected fusion wells are further cloned by dilution until monoclonal cultures producing anti-NgR antibodies are obtained.

B. Humanization of anti-NgR monoclonal antibodies

The expression pattern of NgR as reported herein and the potential of NgRs as targets for therapeutic intervention suggest therapeutic indications for NgR inhibitors (antagonists). NgR-neutralizing antibodies comprise one class of therapeutics useful as
5 NgR antagonists. Following are protocols to improve the utility of anti-NgR monoclonal antibodies as therapeutics in humans by "humanizing" the monoclonal antibodies to improve their serum half-life and render them less immunogenic in human hosts (*i.e.*, to prevent human antibody response to non-human anti-NgR antibodies).

The principles of humanization have been described in the literature and are
10 facilitated by the modular arrangement of antibody proteins. To minimize the possibility of binding complement, a humanized antibody of the IgG4 isotype is preferred.

For example, a level of humanization is achieved by generating chimeric antibodies comprising the variable domains of non-human antibody proteins of interest
15 with the constant domains of human antibody molecules. (See, *e.g.*, Morrison *et al.*, (1989) *Adv. Immunol.*, 44, 65-92). The variable domains of NgR-neutralizing anti-NgR antibodies are cloned from the genomic DNA of a B-cell hybridoma or from cDNA generated from mRNA isolated from the hybridoma of interest. The V region gene fragments are linked to exons encoding human antibody constant domains, and
20 the resultant construct is expressed in suitable mammalian host cells (*e.g.*, myeloma or CHO cells).

To achieve an even greater level of humanization, only those portions of the variable region gene fragments that encode antigen-binding complementarity determining regions ("CDR") of the non-human monoclonal antibody genes are cloned
25 into human antibody sequences. (See, *e.g.*, Jones *et al.*, (1986) *Nature* 321, 522-525; Riechmann *et al.*, (1988) *Nature* 332, 323-327; Verhoeyen *et al.*, (1988) *Science* 239, 1534-1536 ; and Tempest *et al.*, (1991) *Bio/Technology* 9, 266-271). If necessary, the β -sheet framework of the human antibody surrounding the CDR3 regions also is modified to more closely mirror the three dimensional structure of the antigen-binding
30 domain of the original monoclonal antibody. (See Kettleborough *et al.*, (1991) *Protein Engin.* 4, 773-783; and Foote *et al.*, (1992) *J. Mol. Biol.* 224, 487-499).

In an alternative approach, the surface of a non-human monoclonal antibody of interest is humanized by altering selected surface residues of the non-human antibody, e.g., by site-directed mutagenesis, while retaining all of the interior and contacting residues of the non-human antibody. See Padlan (1991) *Mol. Immunol.* 28, 489-498 .

5 The foregoing approaches are employed using NgR-neutralizing anti-NgR monoclonal antibodies and the hybridomas that produce them to generate humanized NgR-neutralizing antibodies useful as therapeutics to treat or palliate conditions wherein NgR expression or ligand-mediated NgR signaling is detrimental.

10 **C. Human NgR-Neutralizing Antibodies from Phage Display**

Human NgR-neutralizing antibodies are generated by phage display techniques such as those described in Aujame *et al.* (1997) *Human Antibodies* 8, 155-168; Hoogenboom (1997) *TIBTECH* 15, 62-70; and Rader *et al.* (1997), *Curr. Opin. Biotechnol.* 8, 503-508, all of which are incorporated by reference. For example,
15 antibody variable regions in the form of Fab fragments or linked single chain Fv fragments are fused to the amino terminus of filamentous phage minor coat protein pIII. Expression of the fusion protein and incorporation thereof into the mature phage coat results in phage particles that present an antibody on their surface and contain the genetic material encoding the antibody. A phage library comprising such constructs is
20 expressed in bacteria, and the library is screened for NgR-specific phage-antibodies using labeled or immobilized NgR as antigen-probe.

D. Human NgR-neutralizing antibodies from transgenic mice

Human NgR-neutralizing antibodies are generated in transgenic mice essentially
25 as described in Bruggemann *et al.* (1996) *Immunol. Today* 17, 391-397 and Bruggemann *et al.* (1997) *Curr. Opin. Biotechnol.* 8, 455-458. Transgenic mice carrying human V-gene segments in germline configuration and that express these transgenes in their lymphoid tissue are immunized with a NgR composition using conventional immunization protocols. hybridomas are generated using B cells from the
30 immunized mice using conventional protocols and screened to identify hybridomas secreting anti-NgR human antibodies (e.g., as described above).

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Example 14: Assays to Identify Modulators of NgR Activity

Set forth below are several nonlimiting assays for identifying modulators (agonists and antagonists) of NgR activity. Among the modulators that can be identified by these assays are natural ligand compounds of the receptor; synthetic analogs and derivatives of natural ligands; antibodies, antibody fragments, and/or antibody-like compounds derived from natural antibodies or from antibody-like combinatorial libraries; and/or synthetic compounds identified by high-throughput screening of libraries; and the like. All modulators that bind NgR are useful for identifying NgR in tissue samples (*e.g.*, for diagnostic purposes, pathological purposes, and the like). Agonist and antagonist modulators are useful for up-regulating and down-regulating NgR activity, respectively, to treat disease states characterized by abnormal levels of NgR activity. The assays may be performed using single putative modulators, and/or may be performed using a known agonist in combination with candidate antagonists (or *visa versa*).

A. cAMP Assays

In one type of assay, levels of cyclic adenosine monophosphate (cAMP) are measured in NgR-transfected cells that have been exposed to candidate modulator compounds. Protocols for cAMP assays have been described in the literature. (See, *e.g.*, Sutherland *et al.*, (1968) *Circulation* 37, 279; Frandsen *et al.*, (1976) *Life Sciences* 18, 529-541; Dooley *et al.*, (1997) *J. Pharmacol. Exp. Therap.* 283, 735-41; and George *et al.*, (1997) *J. Biomol. Screening* 2, 235-40). An exemplary protocol for such an assay, using an Adenylyl Cyclase Activation FlashPlate® Assay from NEN™ Life Science Products, is set forth below.

Briefly, the NgR coding sequence (*e.g.*, a cDNA or intronless genomic DNA) is subcloned into a commercial expression vector, such as pzeoSV2 (Invitrogen), and transiently transfected into Chinese Hamster Ovary (CHO) cells using known methods, such as the transfection protocol provided by Boehringer-Mannheim when supplying the FuGENE 6 transfection reagent. Transfected CHO cells are seeded into 96-well microplates from the FlashPlate® assay kit, which are coated with solid scintillant to which antisera to cAMP has been bound. For a control, some wells are seeded with

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wild type (untransfected) CHO cells. Other wells in the plate receive various amounts of a cAMP standard solution for use in creating a standard curve.

One or more test compounds (*i.e.*, candidate modulators) are added to the cells in each well, with water and/or compound-free medium/diluent serving as a control or controls. After treatment, cAMP is allowed to accumulate in the cells for exactly 15 minutes at room temperature. The assay is terminated by the addition of lysis buffer containing [¹²⁵I]-labeled cAMP, and the plate is counted using a Packard Topcount™ 96-well microplate scintillation counter. Unlabeled cAMP from the lysed cells (or from standards) and fixed amounts of [¹²⁵I]-cAMP compete for antibody bound to the plate. A standard curve is constructed, and cAMP values for the unknowns are obtained by interpolation. Changes in intracellular cAMP levels of cells in response to exposure to a test compound are indicative of NgR modulating activity. Modulators that act as agonists of receptors which couple to the G_s subtype of G proteins will stimulate production of cAMP, leading to a measurable 3-10 fold increase in cAMP levels. Agonists of receptors which couple to the G_{i/o} subtype of G proteins will inhibit forskolin-stimulated cAMP production, leading to a measurable decrease in cAMP levels of 50-100%. Modulators that act as inverse agonists will reverse these effects at receptors that are either constitutively active or activated by known agonists.

20 **B. Aequorin Assays**

In another assay, cells (*e.g.*, CHO cells) are transiently co-transfected with both a NgR expression construct and a construct that encodes the photoprotein apoaequorin. In the presence of the cofactor coelenterazine, apoaequorin will emit a measurable luminescence that is proportional to the amount of intracellular (cytoplasmic) free calcium. (See generally, Cobbold, *et al.* "Aequorin measurements of cytoplasmic free calcium," *In*: McCormack J.G. and Cobbold P.H., eds., CELLULAR CALCIUM: A PRACTICAL APPROACH. Oxford: IRL Press (1991); Stables *et al.*, (1997) *Anal. Biochem.* 252, 115-26; and Haugland, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS. Sixth edition. Molecular Probes, Eugene, OR (1996)).

30 In one exemplary assay, NgR is subcloned into the commercial expression vector pzeoSV2 (Invitrogen) and transiently co-transfected along with a construct that encodes the photoprotein apoaequorin (Molecular Probes, Eugene, OR) into CHO cells

using the transfection reagent FuGENE 6 (Boehringer-Mannheim) and the transfection protocol provided in the product insert.

The cells are cultured for 24 hours at 37°C in MEM (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin and 10 µg/ml streptomycin, at which time the medium is changed to serum-free MEM containing 5 µM coelenterazine (Molecular Probes, Eugene, OR). Culturing is then continued for two additional hours at 37°C. Subsequently, cells are detached from the plate using VERSEN (Gibco/BRL), washed, and resuspended at 200,000 cells/ml in serum-free MEM.

Dilutions of candidate NgR modulator compounds are prepared in serum-free MEM and dispensed into wells of an opaque 96-well assay plate at 50 µl/well. Plates are then loaded onto an MLX microtiter plate luminometer (Dynex Technologies, Inc., Chantilly, VA). The instrument is programmed to dispense 50 µl cell suspensions into each well, one well at a time, and immediately read luminescence for 15 seconds.

Dose-response curves for the candidate modulators are constructed using the area under the curve for each light signal peak. Data are analyzed with SlideWrite, using the equation for a one-site ligand, and EC₅₀ values are obtained. Changes in luminescence caused by the compounds are considered indicative of modulatory activity. Modulators that act as agonists at receptors which couple to the G_q subtype of G proteins give an increase in luminescence of up to 100 fold. Modulators that act as inverse agonists will reverse this effect at receptors that are either constitutively active or activated by known agonists.

C. Luciferase Reporter Gene Assay

The photoprotein luciferase provides another useful tool for assaying for modulators of NgR activity. Cells (*e.g.*, CHO cells or COS 7 cells) are transiently co-transfected with both a NgR expression construct (*e.g.*, NgR in pzeoSV2) and a reporter construct which includes a gene for the luciferase protein downstream from a transcription factor binding site, such as the cAMP-response element (CRE), AP-1, or NF-kappa B. Expression levels of luciferase reflect the activation status of the signaling events. (See generally, George *et al.* (1997) *J. Biomol. Screening* 2, 235-240; and Stratowa *et al.* (1995) *Curr. Opin. Biotechnol.* 6, 574-581). Luciferase

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activity may be quantitatively measured using, *e.g.*, luciferase assay reagents that are commercially available from Promega (Madison, WI).

In one exemplary assay, CHO cells are plated in 24-well culture dishes at a density of 100,000 cells/well one day prior to transfection and cultured at 37°C in
 5 MEM (Gibco/BRL) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin and 10 µg/ml streptomycin. Cells are transiently co-transfected with both a NgR expression construct and a reporter construct containing the luciferase gene. The reporter plasmids CRE-luciferase, AP-1-luciferase and NF-kappaB-luciferase may be purchased from Stratagene (Legally, CA). Transfections are
 10 performed using the FuGENE 6 transfection reagent (Boehringer-Mannheim) according to the supplier's instructions. Cells transfected with the reporter construct alone are used as a control. Twenty-four hours after transfection, cells are washed once with PBS pre-warmed to 37°C. Serum-free MEM is then added to the cells either alone (control) or with one or more candidate modulators and the cells are
 15 incubated at 37°C for five hours. Thereafter, cells are washed once with ice-cold PBS and lysed by the addition of 100 µl of lysis buffer per well from the luciferase assay kit supplied by Promega. After incubation for 15 minutes at room temperature, 15 µl of the lysate is mixed with 50 µl of substrate solution (Promega) in an opaque-white, 96-well plate, and the luminescence is read immediately on a Wallace model 1450
 20 MicroBeta scintillation and luminescence counter (Wallace Instruments, Gaithersburg, MD).

Differences in luminescence in the presence versus the absence of a candidate modulator compound are indicative of modulatory activity. Receptors that are either constitutively active or activated by agonists typically give a 3-20-fold stimulation of
 25 luminescence compared to cells transfected with the reporter gene alone. Modulators that act as inverse agonists will reverse this effect.

D. Intracellular calcium measurement using FLIPR

Changes in intracellular calcium levels are another recognized indicator of
 30 receptor activity, and such assays can be employed to screen for modulators of NgR activity. For example, CHO cells stably transfected with a NgR expression vector are plated at a density of 4×10^4 cells/well in Packard black-walled, 96-well plates

5 specially designed to discriminate fluorescence signals emanating from the various wells on the plate. The cells are incubated for 60 minutes at 37°C in modified Dulbecco's PBS (D-PBS) containing 36 mg/L pyruvate and 1 g/L glucose with the addition of 1% fetal bovine serum and one of four calcium indicator dyes (Fluo-3™ AM, Fluo-4™ AM, Calcium Green™ -1 AM, or Oregon Green™ 488 BAPTA-1 AM), each at a concentration of 4 µM. Plates are washed once with modified D-PBS without 1% fetal bovine serum and incubated for 10 minutes at 37°C to remove residual dye from the cellular membrane. In addition, a series of washes with modified D-PBS without 1% fetal bovine serum is performed immediately prior to activation of the calcium response.

15 A calcium response is initiated by the addition of one or more candidate receptor agonist compounds, calcium ionophore A23187 (10 µM; positive control), or ATP (4 µM; positive control). Fluorescence is measured by Molecular Device's FLIPR with an argon laser (excitation at 488 nm). (See, *e.g.*, Kuntzweiler *et al.* (1998) *Drug Dev. Res.* 44,14-20). The F-stop for the detector camera is set at 2.5 and the length of exposure is 0.4 milliseconds. Basal fluorescence of cells is measured for 20 seconds prior to addition of candidate agonist, ATP, or A23187, and the basal fluorescence level is subtracted from the response signal. The calcium signal is measured for approximately 200 seconds, taking readings every two seconds. Calcium ionophore A23187 and ATP increase the calcium signal 200% above baseline levels. In general, activated NgRs increase the calcium signal at least about 10-15% above baseline signal.

E. [³⁵S]GTPγS Binding Assay

25 It is also possible to evaluate whether NgR signals through a G protein-mediated pathway. Because G protein-coupled receptors signal through intracellular G proteins whose activity involves GTP binding and hydrolysis to yield bound GDP, measurement of binding of the non-hydrolyzable GTP analog [³⁵S]-GTPγS in the presence and absence of candidate modulators provides another assay for modulator activity. (See, *e.g.*, Kowal *et al.*, (1998) *Neuropharmacology* 37, 179-187.).

In one exemplary assay, cells stably transfected with a NgR expression vector are grown in 10 cm tissue culture dishes to subconfluence, rinsed once with 5 ml of ice-cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline, and scraped into 5 ml of the same buffer. Cells are pelleted by centrifugation (500 x g, 5 minutes), resuspended in TEE buffer (25 mM Tris, pH 7.5, 5 mM EDTA, 5 mM EGTA), and frozen in liquid nitrogen. After thawing, the cells are homogenized using a Dounce homogenizer (1 ml TEE per plate of cells), and centrifuged at 1,000 x g for 5 minutes to remove nuclei and unbroken cells.

The homogenate supernatant is centrifuged at 20,000 x g for 20 minutes to isolate the membrane fraction, and the membrane pellet is washed once with TEE and resuspended in binding buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA). The resuspended membranes can be frozen in liquid nitrogen and stored at -70°C until use.

Aliquots of cell membranes prepared as described above and stored at -70°C are thawed, homogenized, and diluted into buffer containing 20 mM HEPES, 10 mM MgCl_2 , 1 mM EDTA, 120 mM NaCl, 10 μM GDP, and 0.2 mM ascorbate, at a concentration of 10-50 $\mu\text{g}/\text{ml}$. In a final volume of 90 μl , homogenates are incubated with varying concentrations of candidate modulator compounds or 100 μM GTP for 30 minutes at 30°C and then placed on ice. To each sample, 10 μl guanosine 5'-O-(3 ^{35}S)thio triphosphate (NEN, 1200 Ci/mmol; [^{35}S]-GTP γS), was added to a final concentration of 100-200 pM. Samples are incubated at 30°C for an additional 30 minutes, 1 ml of 10 mM HEPES, pH 7.4, 10 mM MgCl_2 , at 4°C is added and the reaction is stopped by filtration.

Samples are filtered over Whatman GF/B filters and the filters are washed with 20 ml ice-cold 10 mM HEPES, pH 7.4, 10 mM MgCl_2 . Filters are counted by liquid scintillation spectroscopy. Nonspecific binding of [^{35}S]-GTP γS is measured in the presence of 100 μM GTP and subtracted from the total. Compounds are selected that modulate the amount of [^{35}S]-GTP γS binding in the cells, compared to untransfected control cells. Activation of receptors by agonists gives up to a five-fold increase in [^{35}S]-GTP γS binding. This response is blocked by antagonists.

F. [^3H]Arachidonic Acid Release

The activation of NgRs may also potentiate arachidonic acid release in cells, providing yet another useful assay for modulators of NgR activity. (See, *e.g.*, Kanterman *et al.*, (1991) *Mol. Pharmacol.* 39,364-369.) For example, CHO cells that are stably transfected with a NgR expression vector are plated in 24-well plates at a density of 15,000 cells/well and grown in MEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin and 10 µg/ml streptomycin for 48 hours at 37°C before use. Cells of each well are labeled by incubation with [³H]-arachidonic acid (Amersham Corp., 210 Ci/mmol) at 0.5 µCi/ml in 1 ml MEM supplemented with 10 mM HEPES, pH 7.5, and 0.5% fatty-acid-free bovine serum albumin for 2 hours at 37°C. The cells are then washed twice with 1 ml of the same buffer.

Candidate modulator compounds are added in 1 ml of the same buffer, either alone or with 10 µM ATP and the cells are incubated at 37°C for 30 minutes. Buffer alone and mock-transfected cells are used as controls. Samples (0.5 ml) from each well are counted by liquid scintillation spectroscopy. Agonists which activate the receptor will lead to potentiation of the ATP-stimulated release of [³H]-arachidonic acid. This potentiation is blocked by antagonists.

G. Extracellular Acidification Rate

In yet another assay, the effects of candidate modulators of NgR activity are assayed by monitoring extracellular changes in pH induced by the test compounds (see, *e.g.*, Dunlop *et al.* (1998) *J. Pharmacol. Toxicol. Meth.* 40, 47-55). In one embodiment, CHO cells transfected with a NgR expression vector are seeded into 12 mm capsule cups (Molecular Devices Corp.) at 4×10^5 cells/cup in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin. The cells are incubated in this medium at 37°C in 5% CO₂ for 24 hours.

Extracellular acidification rates are measured using a Cytosensor microphysiometer (Molecular Devices Corp.). The capsule cups are loaded into the sensor chambers of the microphysiometer and the chambers are perfused with running buffer (bicarbonate-free MEM supplemented with 4 mM L-glutamine, 10 units/ml penicillin, 10 µg/ml streptomycin, 26 mM NaCl) at a flow rate of 100 µl/minute.

Candidate agonists or other agents are diluted into the running buffer and perfused through a second fluid path. During each 60-second pump cycle, the pump is run for 38 seconds and is off for the remaining 22 seconds. The pH of the running buffer in the sensor chamber is recorded during the cycle from 43-58 seconds, and the pump is re-started at 60 seconds to start the next cycle. The rate of acidification of the running buffer during the recording time is calculated by the Cytosoft program. Changes in the rate of acidification are calculated by subtracting the baseline value (the average of 4 rate measurements immediately before addition of a modulator candidate) from the highest rate measurement obtained after addition of a modulator candidate. The selected instrument detects 61 mV/pH unit. Modulators that act as agonists of the receptor result in an increase in the rate of extracellular acidification compared to the rate in the absence of agonist. This response is blocked by modulators which act as antagonists of the receptor.

Example 15: mNgR3 does not bind hNogo-A(1055-1120)

To functionally test the mouse NgR3 (hereinafter, mNgR3) for its ability to bind hNogo-A(1055-1120), a cDNA expression vector for a myc epitope-tagged mNgR3 protein was created. The mouse NgR3 cDNA was amplified by PCR from mouse adult brain cDNA, from the signal sequence to the stop codon, and ligated into the pSecTag2 vector such that the vector encodes a signal sequence followed by a myc tag followed by the mature mNgR3 sequence. This plasmid was transfected into COS07 cells, and expression of a myc-tagged protein of the predicted size was verified by immunoblot analysis. Alkaline phosphatase-hNogo-A(1055-1120) binding studies and myc immunohistology were conducted as described (Fournier et al., supra).

The cells expressing mNgR3 express the myc-tagged protein but binding to AP-hNogo-A(1055-1120) was not observed under the conditions employed (Fig. 8).

Example 16: Identification of partial human NgR3 cDNA and protein sequences

The tblastn program was used to search for the human homolog of mouse NgR3. The mouse NgR3 protein sequence (SEQ ID NO:4) was used to query a proprietary human expressed sequence tag (EST) database from Incyte yielding one highly significant hit: Incyte Template ID 190989.1. This sequence (937 nucleotides)

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contains an open reading frame of 312 amino acids in the second reverse frame that exhibits 88% identity with residues 66 to 381 of mouse NgR3 (SEQ ID NO:4), strongly indicating that it is part of the human NgR3 homolog.

A query of SEQ ID NO:4 against the public human EST database in Genbank
5 also produced a hit with a 465-bp EST (Accession number: R35699; Version number: R35699.1; GI: 792600). There are a number of single nucleotide deletions and insertions within this sequence which cause frame shift errors. All of the reliable sequence contained in this public EST is present in the Incyte EST (Template ID 190989.1).

10 To obtain more nucleotide sequence that would extend the amino acid sequence at that carboxy terminal end, the I.M.A.G.E. Consortium clone No. 38319, which corresponds to Genbank accession No. R35699, was purchased from Incyte Genomics Inc. and subjected to further DNA sequence analysis. This clone consists of a NotI/HinD III fragment containing the sequence of interest, cloned into the
15 NotI/HinD III sites of the vector Lafmid BA (<http://image.llnl.gov/image/html/libs/lafmidBA.shtml>). The clone was received as an agar stab, which was streaked out on LB agar plates containing 50ug/ml ampicillin to isolate individual colonies. Six colonies were grown in LB medium with antibiotic, and plasmid DNA was prepared using the Promega Wizard Plus Miniprep DNA
20 Purification System (Promega #A7500). These DNAs were subsequently digested with NotI and HinD III restriction enzymes to confirm that the clones contained an insert. The insert of one isolate was sequenced using a combination of vector specific and gene specific primers yielding a partial nucleotide sequence of human NgR3 of 1176 nucleotides (SEQ ID NO:13). A translation of this sequence provides a partial
25 sequence for human NgR3 of 392 amino acids (SEQ ID NO:14).

The nucleotide sequence of SEQ ID NO:13 differs from the Incyte EST sequence at three positions. Nucleotide positions 12-13 in SEQ ID NO:13 are CG, whereas the corresponding nucleotides in the Incyte Template ID 190989.1 are GT (i.e., positions 12-13 of the complement of Incyte Template ID 190989.1). In
30 addition, position 641 in SEQ ID NO:13 is a C, whereas the corresponding nucleotide in the Incyte Template ID 190989.1 sequence is an A (i.e., position 641 of the complement of Incyte Template ID 190989.1). This results in two changes in amino

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acids when comparing SEQ ID NO:14 to the ORF encoded by Incyte Template 190989.1: SEQ ID NO:14 contains a valine at position 5, whereas the ORF encoded by Incyte Template ID 190989.1 contains a leucine; SEQ ID NO:14 contains an alanine at position 214, whereas the ORF encoded by Incyte Template ID 190989.1 contains a glutamic acid.

The nucleotide sequence of SEQ ID NO:13 differs from the public EST (Accession number: R35699; Version number: R35699.1; GI: 792600) sequence at two positions (within the first 200 nucleotides of reliable sequence). Nucleotide positions 12-13 in SEQ ID NO:13 are CG, whereas the corresponding nucleotides in the public EST are GT (i.e., positions 12-13 of the public EST; Accession no: R35699; Version no: R35699.1; GI: 792600) This leads to a single amino acid change when comparing SEQ ID NO:14 to the ORF encoded by the public EST: SEQ ID NO:14 contains a valine at position 5, while the ORF encoded by the public EST contains a leucine.

A Bestfit analysis of the partial human amino acid sequence with the full-length mouse amino acid sequence indicates that the human NgR3 amino acid sequence is complete at the carboxy terminal end and that they share 89.54% identity. An alignment of all the NgR proteins is shown in Figure 9. Although the human NgR3 amino acid sequence is missing the first 25 amino acids, it can be determined that the human NgR3 protein contains the following features in common with the other NgR sequences: (1) eight Leucine Rich Repeat (LRR) domains; (2) an LRR carboxy-terminal (LRR-CT) domain; (3) a conserved cysteine in the fourth LRR domain; (4) a conserved potential glycosylation site in the eighth LRR domain; and (5) a hydrophobic carboxyl terminus.

As those skilled in the art will appreciate, numerous changes and modifications may be made to the preferred embodiments of the invention without departing from the spirit of the invention. It is intended that all such variations fall within the scope of the invention.

The entire disclosure of each publication cited herein is hereby incorporated by reference. This application claims benefit from United States provisional application 60/238,361, filed October 6, 2000, which is incorporated by reference herein in its entirety.

Key for Sequence Listing:

	SEQ ID NO:1	human NgR2 cDNA sequence derived from genomic sequence AC013606
	SEQ ID NO:2	human NgR2 amino acid sequence
5	SEQ ID NO:3	mouse NgR3 cDNA sequence derived from AC021768
	SEQ ID NO:4	a mouse NgR3 amino acid sequence
	SEQ ID NO:5	a human NgR1 amino acid sequence
	SEQ ID NO:6	a consensus amino acid sequence for NgRs
	SEQ ID NO:7	#1055-1120 amino acid residues of hNogoA (Nogo-66)
10	SEQ ID NO:8	a mature human NgR2 amino acid sequence
	SEQ ID NO:9	a mature mouse NgR3 amino acid sequence
	SEQ ID NO:10	a consensus NgR LLRNT amino acid sequence
	SEQ ID NO:11	a consensus NgR LRRCT domain amino acid sequence
	SEQ ID NO:12	a consensus NgR LRR domain amino acid sequence
15	SEQ ID NO:13	a partial human NgR3 nucleotide sequence
	SEQ ID NO:14	a partial human NgR3 amino acid sequence
	SEQ ID NO:15	a genomic sequence encoding a human NgR2 sequence.
	SEQ ID NO:16	a genomic sequence (complementary strand) encoding a mouse NgR3
20	SEQ ID NO:17	a mouse NgR1 amino acid sequence
	SEQ ID NO:18	a consensus sequence for the NTLRRCT domain of NgR
	SEQ ID NO:19	an consensus NgR LRRCT domain amino acid sequence

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